EUROPEAN COMMISSION
Research & Innovation

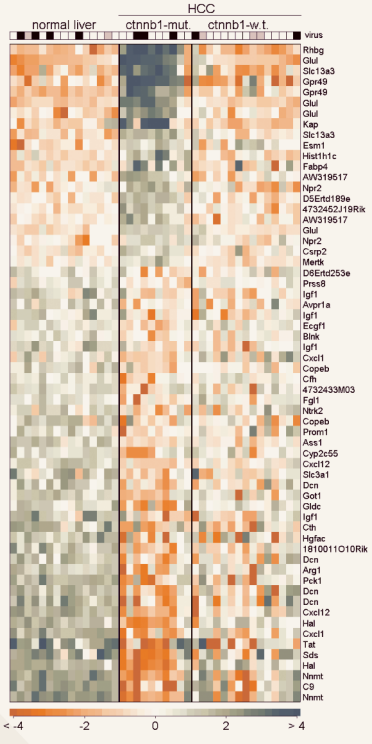
Cosmetics Europe
the personal care association

SEURAT-1

Paracelsus (1493-1541):
"The dose makes the poison"

Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity Testing

Toxicology in the 21st century:
Mechanism-driven Toxicology
defines the safe dose



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Health Programme: Advanced therapies and systems medicine (F4)
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Paracelsus (1493 - 1541)
Portrait by Quentin Massys

« The dose makes the poison »

Paracelsus was a 16th century physician and alchemist who made significant progress in the field of medicine. Pioneer in chemistry, he made revolutionary advances in understanding and treating wounds and diseases.

Vol. 1 Vol. 2 Vol. 3 Vol. 4 Vol. 5 Vol. 6

This is the third out of six annual volumes describing scientific progress, strategic development and evolution of the legislative and regulatory context in the field of repeated dose systemic toxicity.

The picture series illustrates the phase-out and replacement of the classical concepts of Toxicology.

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"Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals"

Implementation of the Research Strategy

volume 3

Edited by:
Tilman Gocht, Michael Schwarz

SEURAT-1

SEURAT was announced as a strategy of the FP7 Health Theme by director Dr. Manuel Hallen on the occasion of the EPAA Annual Conference in 2008 in line with Commissioners G. Verheugen and J. Potocnik. The long term strategic target is defined as "Safety Evaluation Ultimately Replacing Animal Testing" (SEURAT).

SEURAT-1 is the Research Initiative launched by the European Commission and the European Cosmetics Association Colipa (funding: EUR 50 million from 2011 to 2015). It is called "SEURAT-1", indicating that more steps have to be taken before the final strategic target will be reached. **SEURAT-1** will develop a long term research strategy and building blocks needed for the development of new non-animal test systems in the field of repeated dose systemic toxicity for the innovative assessment of human safety.

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Foreword

There are important scientific, ethical, financial and legislative reasons why there should be a continuing commitment to the development of methods that seek to reduce, refine, and ultimately replace, the use of animals in pure and applied research.

One area in which there has been a very substantial investment is the development of alternative methods has been toxicology/safety assessment. There have been some successes, and for certain aspects of safety assessment validated methods are available, or are in late stage development. However, in other areas of toxicology and predictive testing there remain substantial challenges. Predictive toxicology needs to be alert to the unexpected. The classical reliance on the use of animals reflects the substantial hurdles that exist in recapitulating holistically *in vitro* or *in silico* the complexity of a living organism and the many integrated cellular and molecular processes, and physiological systems, that might represent potential targets for toxicants.

The challenge reaches its apotheosis in consideration of whether continued low level exposure to a chemical or other xenobiotics will result in adverse health effects in humans. In theory at least a chemical or drug could perturb one or more of many cellular and molecular systems that, in turn, might drive the development of important adverse health effects. How then can this be explored and an appropriate safety assessment performed. The solution currently is the repeat dose systemic toxicity study in animals. The big prize in predictive toxicology would be to design an alternative that did not require the use of animals.

The only way this is going to be achieved, and truly useful alternative methods for hazard and risk assessment developed successfully, is if we can harness expertly and exploit fully the best that science and technology has to offer in designing new strategies.

Working towards that goal is the primary focus of **SEURAT-1** (*Safety Evaluation Ultimately Replacing Animal Testing)*. This is a substantial 5 year pan-European programme, comprising a large number of research institutions and companies, and supported through an award of €50M made available jointly by the European cosmetics industry and the European Commission.

The programme was launched formally at the beginning of 2011 and this is the 3rd book summarising progress to date. The objectives are laudable and described here are the efforts that the **SEURAT-1** programme is making to achieve those goals.

Professor Ian Kimber

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Executive Summary

On 11 March 2013 the full ban on animal testing for cosmetic products entered into force. From this date, animal testing for marketing of new cosmetic products in the European Union is prohibited. This deadline was set by the Seventh Amendment of the Council Directive on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC, 'Cosmetics Directive') and triggered the establishment of a European Research Initiative in the field of repeated dose systemic toxicity.

This publication is the third volume of a series of six Annual Reports that summarise the activities of the **SEURAT-1** Research Initiative. **SEURAT-1** works towards the long-term chemical safety testing target of 'Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT), which was presented by the FP7 HEALTH theme in 2008. The framework for this Research Initiative was created in June 2009 through the FP7 call for proposals 'Alternative Testing Strategies: Towards the replacement of *in vivo* repeated dose systemic toxicity testing' with a total funding of EUR 50 million. It is called '**SEURAT-1**', indicating that this is the first step in the specific area of repeated dose systemic toxicity addressing the global long-term strategic target of SEURAT. The **SEURAT-1** Research Initiative started on 1 January 2011 and is co-funded by the European Commission Directorate-General for Research & Innovation within the HEALTH theme of the Seventh European Research Programme (FP7) and Cosmetics Europe.

The aim of the **SEURAT-1** Research Initiative is the development of a long-term research strategy leading to pathway-based human safety assessments in the field of repeated dose systemic toxicity testing of chemicals. The overall goal is to establish animal-free Innovative Toxicity Testing (ITT) methods, enabling robust safety assessments that are more predictive than existing testing procedures. In order to achieve this, a cluster of five research projects spread over 70 European universities, public research institutes and private companies has been organised, supported by a 'data handling and servicing project' and a 'coordination and support project'. The Scientific Expert Panel, which is composed of the **SEURAT-1** project coordinators and external international experts in the field of repeated dose systemic toxicity, provides scientific advice regarding the research work and future direction of the **SEURAT-1** Research Initiative and, thus, plays a key role in its scientific coordination.

Objectives of the SEURAT-1 Research Initiative
Develop highly innovative tools and methodology that can ultimately support regulatory safety assessment
Formulate and implement a research strategy based on generating and applying knowledge of mode-of-action
Demonstrate proof-of-concept at multiple levels - theoretical, systems and application
Provide the blueprint for expanding the applicability domains - chemical, toxicological and regulatory

The **SEURAT-1** Research Initiative combines expertise in cell culture for the preparation of stable human cell lines with the establishment of sophisticated experimental systems such as organ simulating devices. The experimental work is linked with advanced methods of computational modelling and estimation techniques, taking innovative systems biology approaches into consideration, requiring a coordinated effort from the **SEURAT-1** Research Initiative. The focal point of these joint activities is given by proof-of-concept studies (case studies) on three levels, demonstrating that (i) mode-of-action theory provides a solid foundation for mechanistic understanding of adverse effects at the subcellular scale (theoretical level), which (ii) can be converted into the development of animal-free Innovative Toxicity Testing methods (product level) that will (iii) ultimately support regulatory safety assessment (application level). The achievement of these proof-of-concept studies forms the backbone for the **SEURAT-1** roadmap (*Figure 1*), which was developed based on key contributions from each of the projects addressing the cluster-level objectives, and created by the coordination action project COACH in close cooperation with the project coordinators, and subsequently endorsed by the Scientific Expert Panel. It is as yet impossible to cover all toxicological endpoints with such a strategy, but the mechanism-based **SEURAT-1** case study approach is designed to provide a cornerstone in the transition from descriptive to predictive toxicology.

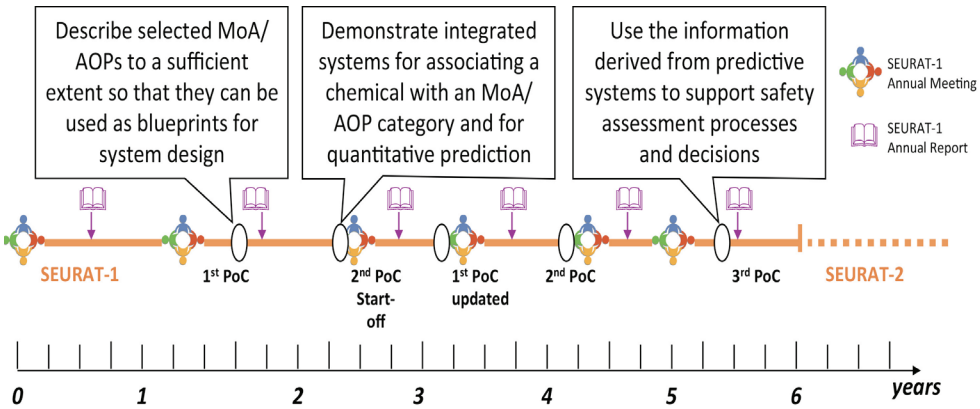

Figure 1 *Roadmap illustrating the timing of the proof-of-concept (PoC) at three conceptual levels as the backbone for interactions between the* ***SEURAT-1*** *projects.*

The infrastructure for such a collaborative, interactive task has been established through the organisation of cross-cluster working groups focusing on: (i) the selection of standard reference compounds to be used for toxicity testing (Gold Compounds Working Group); (ii) the data exchange between the projects and the standardisation of data analysis (Data Analysis Working Group); (iii) the identification of modes-of-action relevant for repeated dose systemic toxicity (Mode-of-Action Working Group); (iv) the *in vitro* to *in vivo* extrapolation and the calculation of appropriate concentration ranges to be tested in *in vitro* experiments (Biokinetics Working Group); (v) the standardisation of quality control issues of the cells used by the different partners and projects (Stem Cells Working Group); and (vi) bridging the gap between non-animal toxicity testing and the safety assessment decision-making needs (Safety Assessment Working Group).

This third Annual Report, prepared by the coordination and support action project COACH, presents a comprehensive overview of research highlights from the different projects of the **SEURAT-1** Research Initiative. This is given in the context of recent developments in European legislation regarding the regulation of chemicals to improve safety assessment and related international activities.

Chapter 1 provides a general introduction to the **SEURAT-1** Research Initiative. It describes the history of the call for research proposals under FP7, and key elements of the upcoming new European Framework Programme for Research and Innovation (Horizon 2020) and how a possible SEURAT-2 phase would fit into this. Furthermore, it provides an overview of the cluster-level objectives as well as the structure and organisation of the **SEURAT-1** Research Initiative.

Chapter 2 outlines the context of the **SEURAT-1** Research Initiative from various perspectives, as follows:

(i) Legislation: On 11 March 2013 the full ban on animal testing for cosmetic products came into force, despite the fact that alternative methods to animal testing are not available for a number of endpoints. The rationale of the implementation of the testing ban for cosmetic products is discussed in the context of its history. The consequences are not yet clear, but there is no doubt that this date marks an important cornerstone for the **SEURAT-1** Research Initiative, as the needs for the development of *in vitro* approaches for the replacement of *in vivo* testing methods have never been greater than today.

(ii) Regulation: The results from the different research programmes focusing on the development of animal-free, innovative testing strategies are becoming more readily available. However, it is still unclear how such strategies may be implemented in future safety assessment approaches. Outlined in this Annual Report is a pragmatic proposal for a tiered toxicity testing and safety assessment framework that takes into account the scientific progress in, as well as the uncertainty of, *in vitro* testing methods and use of non-standard data for the reduction of animal testing. The framework, reflecting to some extent the dynamic nature of the interface between science and regulation, could be implemented today into safety assessment and would be, with increased knowledge, adaptable to scientific progress.

(iii) Science: This year's Annual Report highlights the importance of biokinetics in the context of developing *in vitro* testing methods and briefly introduces computational methods for predicting molecular initiating events. As stated above, the **SEURAT-1** Research Initiative targets the development of highly innovative tools and methodologies that can ultimately support regulatory safety assessment without animal testing. Hence, quantitative *in vitro* to *in vivo* extrapolation will be key to the acceptance and application of **SEURAT-1** methods in regulatory safety assessment. In this section, theoretical considerations are combined with practical advice about what needs to be taken into account when designing laboratory studies addressing the prediction of repeated dose systemic toxicity (e.g., development of a dosing protocol, etc.). Furthermore, as **SEURAT-1** follows a mode-of-action driven strategy (see below), the use of toxicity databases to gain insights into molecular initiating events is discussed.

Chapter 3 focuses on the development of a long-term research strategy and its implementation within the cluster. As already outlined in the first and second Annual Reports, the research

strategy is to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure needed for safety assessment. Proof-of-concept studies should demonstrate the feasibility that knowledge about modes-of-action really can be converted into the development of integrated testing strategies to be used for safety assessment, and this third Annual Report focuses on the design of these proof-of-concept studies. Initial descriptions of adverse outcome pathways were prepared following OECD guidance and the associated development template. Key events within these adverse outcome pathways are currently being characterised, as they will be the points of reference for assay development within the **SEURAT-1** Research Initiative. Testing methods developed in **SEURAT-1** will be based on mechanistic understanding and should be predictive within a set of pre-defined goals that are currently being developed. The integration of different testing methods under the umbrella of the adverse outcome pathway concept will then lead to the formulation of case studies, demonstrating that **SEURAT-1** methods can indeed support regulatory safety assessment. Two different categories of case studies reflecting two typical safety assessment scenarios will be addressed: (i) the objective of the first case study is to arrive at a point of departure for a particular chemical, which can be used as a basis for safety decision by conducting an *ab initio* assessment using the new methods developed within the **SEURAT-1** Research Initiative; and (ii) the objective of the second case study is to use the **SEURAT-1** methods in the context of 'read-across', that is, to demonstrate how information about a chemical generated using **SEURAT-1** methods can be used to associate it with a chemical category, and to predict its hazardous properties by 'reading across' from the properties of other chemicals belonging to the same category. The definition and execution of the case studies on all three proof-of-concept levels (theoretical level: description of modes-of-action; systems level: development of integrated testing strategies; application level: use of these methods to support safety assessment) is highly inclusive, in that the partners, research projects, working groups, the **SEURAT-1** Scientific Expert Panel and industry advisers are all involved and contributing to the process.

Mode-of-Action descriptions being developed within SEURAT-1
From protein alkylation to liver fibrosis
From Liver X Receptor activation to liver steatosis
From inhibition of the bile salt export pump to cholestasis
From NMDA-R over activation to neurodegeneration
From NMDA-R inactivation to disturbed network formation
From NTE inhibition to Wallerian degeneration
From GABA-R blocking to altered neuronal network function

The detailed project descriptions and their research highlights from the second year are given in chapter 4. The **SEURAT-1** Research Initiative is designed as a coordinated cluster of five research projects, supported by a 'data handling and servicing project' and a 'coordination and support project'. The tasks of the projects and the highlights presented in this Annual Report are:

Stem cell differentiation for providing human-based organ specific target cells to assay toxicity pathways *in vitro*.

The *SCR&Tox* report focuses on the adaptation of bioelectronic measurement systems for label-free monitoring of cell functionality as a basis for toxicity testing. Multielectrode array-based bioelectronic monitoring platforms were optimised for recording the cardiac electrophysiological functionality of human cardiomyocyte clusters. Furthermore, highly matured and electrophysiologically active neuronal networks could be established on an microelectrode array-based monitoring platform, offering the opportunity to observe neurotoxicity by functional bioelectronic monitoring over days or even weeks.

Development of a hepatic microfluidic bioreactor mimicking the complex structure and function of the human liver.

HeMiBio reports on progress in the phenotypic characterisation of cells to be incorporated in the bioreactor. Furthermore, master pluripotent stem cell (PSC) lines suitable for recombinase-mediated cassette exchange were generated, allowing creation of cells wherein molecular sensors for toxicity testing can be incorporated. External sensors based on optical and electrochemical detection techniques were characterised and incorporated in initial prototype bioreactors.

Identification and investigation of human biomarkers in cellular models for repeated dose *in vitro* testing.

DETECTIVE has optimised standard operating procedures and collected data across a variety of different cellular models, including human iPSC-derived cardiomyocytes, primary human hepatocytes and renal epithelial cells. The cardiomyocytes can be maintained in good culture conditions for up to 14 days, giving a window for long-term repeated dose toxicity studies. The transcriptomic and proteomic screenings for identification of molecular mechanisms of toxicity in *in vitro* systems of primary human hepatocytes revealed hundreds of genes and proteins that are severely affected by exposure to acetaminophen at a toxic concentration. Further-

more, a panel of GFP-tagged HepG2 reporter cell lines that focus on many adaptive stress-response pathways has been constructed. Finally, a fully integrated '-omics' analysis of two compounds for the kidney model was completed in the second year.

COSMOS

Delivery of an integrated suite of computational tools to predict the effects of long-term exposure to chemicals in humans based on *in silico* calculations.

COSMOS is developing molecular modelling techniques for modes-of-action that involve receptor binding as a key event, which is relevant for the mode-of-action 'From Liver X Receptor activation to liver steatosis' (see text box). Experimental information on available structure-activity relationship data and three-dimensional models of LXR were retrieved and analysed as a basis for modelling LXR-binding affinity. A second key aspect of COSMOS activities in the second year was in the field of quantitative *in vitro* to *in vivo* extrapolation through coupling of a PBPK model with a multi-scale model representing the liver.

NOTOX

Development of systems biology tools for organotypic human cell cultures suitable for long-term toxicity testing, and the identification and analysis of pathways of toxicological relevance.

NOTOX has focused on the characterisation of 3D organotypic cultures of HepaRG as a tool for *in vitro* toxicity testing. The organotypic cultures maintained hepatocyte-specific functions over a three-week cultivation period, including (i) production of albumin, urea and glucose, and (ii) phase I, II and III enzyme activities. Initial acute toxicity studies were conducted and revealed the suitability of the test system to study drug-induced hepatotoxicity. NOTOX successfully implemented a mathematical model based on cellular drug metabolism, generation of reactive species and the link to the cellular scavenger system of glutathione. This model is a sound basis for future fast and individualised toxicity screening.

ToxBank

Data management, cell and tissue banking, selection of 'reference compounds' and chemical repository.

A preliminary version of the ToxBank Data Warehouse was implemented that will provide access to all experimental, processed data and protocols alongside relevant public information. This includes the development of web-based interfaces for linking and uploading data, including raw and processed data, and model results. A web-based user interface

for searching, browsing and filtering the results was implemented to provide access to all protocols and data across the **SEURAT-1** Research Initiative.

COACH

Cluster-level coordinating and support action.

The COACH report provides information about the cluster-level coordination activities, facilitating exchanges between the projects, and dissemination of research activities at the cluster level.

Chapter 4 also contains reports about the meetings of each of the specific projects as well as of the **SEURAT-1** Research Initiative as a whole. These meetings were conducted to provide input into the annual action plan, as well as to foster collaborations between the projects. A detailed overview of the development of the **SEURAT-1** roadmap, highlighting the contributions of the individual projects to the achievement of cluster-level objectives, is presented in a section describing cross-cluster cooperation. The above-mentioned working groups play a vital role in the effort to make the whole greater than the sum of its parts. Reports on activities and workshops conducted under the umbrella of these working groups are also included in this section, highlighting the fact that the cross-cluster working groups have become the driving force behind cluster-level progress.

Chapter 5 describes the related international activities. The list of short project descriptions that was launched in the first Annual Report and continued in the second has been updated, with special emphasis on initiatives focusing on repeated dose toxicity and the replacement of animal testing in the field of human safety assessment. For **SEURAT-1** to be successful, it is important to collaborate with the various complementary international research programmes on the way 'towards the replacement of *in vivo* repeated dose systemic toxicity testing'. At the time of writing this report COACH is organising a meeting between **SEURAT-1** and the related US initiative Tox21 (25–27 June 2013 in Ispra, Italy). The aim of the meeting is to build real collaborations between the two research initiatives, in order to exchange experiences and knowledge with the possibility of bilaterally contributing to common projects. As the **SEURAT-1** Research Initiative reaches midterm it is time to look forward to and outline the elements of a possible second SEURAT phase. Therefore, some thoughts about the continuation of the SEURAT work programme finalise this third **SEURAT-1** Annual Report.

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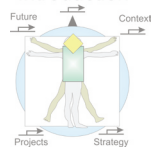


1 INTRODUCTION

'The real voyage of discovery consists not in seeking new landscapes, but in having new eyes.'

Marcel Proust

Background

On 11 March 2013 the full ban on animal testing for cosmetic products came into force. From this date, animal testing for marketing of new cosmetic products in the European Union is prohibited. Data from animal testing that was carried out before the implementation date of the marketing ban can be further used in the safety assessment of cosmetic products. The implementation of the marketing and testing ban follows the Seventh Amendment of the Council Directive on the approximation of the laws of the Member States relating to cosmetic products (76/768/EEC, 'Cosmetics Directive'), which defined the step-wise phase-out of animal testing for cosmetic products as well as for cosmetic ingredients over the last 10 years. Accordingly, animal testing for cosmetic products has already been prohibited since 2004, but the deadline for the most complex fields of repeated dose toxicity, reproductive toxicity and toxicokinetics was extended to 11 March 2013. Although an expert panel of scientists came to the conclusion that they cannot estimate the required time for establishing alternative methods for the full replacement of animal testing in the field of repeated dose systemic toxicity (due to unresolved questions related to the involved complex cellular mechanisms and appropriate biological models that would cover these mechanisms; *Adler et al., 2011*), the deadline was not further extended. In fact,

'*the testing and marketing bans in the Cosmetics Directive/Regulation apply even in case alternative methods to animal testing are not yet available. This reflects a sector-specific political choice by the European Parliament and the Council*.' *(European Commission, 2013)*

Triggered by this legislative pressure, Cosmetics Europe – The Personal Care Association (previously named Colipa) had proposed a contribution of EUR 25 million at the beginning of 2008 to support the research work in the area of repeated dose systemic toxicity. 'Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT) was presented by the HEALTH Theme of the Directorate General of Research and Innovation of the European Commission in 2008 as the long-term target in safety testing. Cosmetics Europe and the European Commission agreed on setting up a Research Initiative for the development of a research strategy 'Towards the replacement of *in vivo* repeated dose systemic toxicity testing'. It was called '**SEURAT-1**', to indicate that this is a first step in a specific area addressing the global long-term strategic target SEURAT. A tiered approach is foreseen, starting with innovative concepts for repeated dose systemic toxicity and ending with animal-free Innovative Toxicity Testing (ITT), enabling robust safety assessment. A model for such a type of joint funding did not exist, but the importance of the proposed research area was evident, in particular because its relevance goes far beyond the requirements of the 'Cosmetics Directive'.

In June 2009 the framework for the **SEURAT-1** Research Initiative was created through a call for proposals under the HEALTH Theme of the 7th European RTD Framework Programme: 'Alternative Testing Strategies: Towards the replacement of *in vivo* repeated dose systemic toxicity testing' with a total budget of EUR 50 million. Cosmetics Europe published its financial

commitment to the Research Initiative at the same time. EUR 25 million funding was provided by the FP7 HEALTH theme and EUR 25 million by Cosmetics Europe.

The **SEURAT-1** Research Initiative started in January 2011. Even though **SEURAT-1** was initially motivated by the urgent needs of the cosmetic industry, it is undoubtedly relevant for other related fields. Systemic toxicity testing is also needed for a variety of applications: in the context of the European Union Regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals); in the development of pharmaceuticals; and in other industrial sectors. Moreover, the scientific knowledge delivered by the **SEURAT-1** Research Initiative is expected to be highly relevant in personalised medicine, systems medicine, in the development of innovative diagnostic tools, in regenerative medicine, and other fields. Therefore, broad impact of the research cluster is expected, bringing the consortium into a leading position internationally in this field of research.

Human Safety Assessment and Horizon 2020

The continuation of the SEURAT programme would be possible under the umbrella of the European Commission's new funding scheme, Horizon 2020. Besides excellent science, Horizon 2020 highlights the priority of industrial leadership and will provide investments in key industrial areas, including biotechnology. Societal challenges are the third priority for future investments under Horizon 2020, reflecting the policy priorities of the Europe 2020 strategy. Major concerns shared by citizens in Europe and elsewhere should be addressed, and, according to the official document describing the new EU Framework Programme (*European Commission, 2011*), the area of 'Health, demographic changes and wellbeing' was identified as one out of six societal challenges on which funding will be focussed. Topics to be addressed include the integration of molecular biological, epidemiological and toxicological approaches as well as the integration of toxicological testing to seek alternatives to animal testing and to improve human safety assessment. Uptake of research activities by the market will be key to the success of applications for funding under Horizon 2020, as a new focus on innovation-related activities bridging the gap between fundamental research, the development of knowledge-driven new products and their implementation into the market will be established.

SEURAT-1 already addresses some important aspects of Horizon 2020. The joint funding by the European Commission and a specific industrial sector (cosmetics industry/Cosmetics Europe) may become a role model, taking into account the aspect of industrial leadership as an important factor for funding under the umbrella of Horizon 2020. Scientifically, **SEURAT-1** aims at a proof-of-concept level, demonstrating that mechanistic understanding of toxicity pathways constitutes a solid basis for a future full implementation based on the development of innovative testing methods that will be more predictive than existing testing procedures. Hence, moving the concepts developed within **SEURAT-1** forward to the industrial scale would be an important topic of a possible SEURAT-2 phase.

Goals and Objectives

The goal of the five-year **SEURAT-1** Research Initiative is to develop a consistent research strategy ready for implementation in the following research programmes. This includes establishing innovative scientific tools for a better understanding of repeated dose toxicity based on *in vitro* tests and identifying gaps in knowledge, which are to be bridged by future research work. The end result will be testing methods which, within the framework of safety assessment, have a higher predictive value, are faster and cheaper than those currently used, and significantly reduce the use of animal tests.

The cluster level objectives, which cannot be achieved by the individual projects alone, are

- to formulate and implement a research strategy based on generating and applying knowledge of mode-of-action;
- to develop highly innovative tools and methodology that can ultimately support regulatory safety assessment;
- to demonstrate proof-of-concept at multiple levels – theoretical, systems and application;
- to provide the blueprint for expanding the applicability domains – chemical, toxicological and regulatory.

The research work in the **SEURAT-1** projects comprises the development of innovative testing strategies, including organ-simulating devices equipped with human-based target cells for toxicity testing, the identification of relevant endpoints and intermediate markers, the application of approaches from systems biology, computational modelling and estimation techniques, and integrated data analysis. Overall, the **SEURAT-1** Research Initiative will contribute significantly to the establishment of a new paradigm in toxicology, which is summarised in the term 'predictive toxicology'.

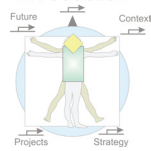
Structure of the **SEURAT-1** Research Initiative

The **SEURAT-1** Research Initiative is designed as a coordinated cluster of five research projects, supported by a 'data handling and servicing project' and a 'coordination and support project' at the cluster level.

The following six projects form the backbone of **SEURAT-1**:

- 'Stem Cells for Relevant efficient extended and normalized TOXicology' (*SCR&Tox*)

  Scientific coordinator: Marc Peschanski, INSERM/UEVE 861, I-STEM/AFM, Evry/France



- 'Hepatic Microfluidic Bioreactor' (*HeMiBio*)

  Scientific coordinator: Catherine Verfaillie, Interdepartmental Stem Cell Institute, Katholieke Universiteit Leuven/Belgium

- Detection of endpoints and biomarkers for repeated dose toxicity using *in vitro* systems' (DETECTIVE)

  Scientific coordinator: Jürgen Hescheler, Institute for Neurophysiology, University Hospital Cologne/Germany

- 'Integrated *In Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety' (COSMOS)

  Scientific coordinator: Mark Cronin, School of Pharmacy and Chemistry, Liverpool John Moores University/United Kingdom

- 'Predicting long term toxic effects using computer models based on systems characterization of organotypic cultures' (NOTOX)

  Scientific coordinator: Elmar Heinzle, Biochemical Engineering, Saarland University, Saarbrücken/Germany

- 'Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology' (ToxBank)

  Scientific coordinator: Barry Hardy, Douglas Connect, Zeiningen/Switzerland

Furthermore, a coordination action project was designed in order to facilitate cluster interaction and activities:

- 'Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals' (COACH)

  Coordinator: Bruno Cucinelli, ARTTIC, Paris/France.

The scientific management and coordination of the **SEURAT-1** Research Initiative is strongly supported by the Scientific Expert Panel (SEP), which plays a key role in providing scientific advice regarding the research work and future orientation of **SEURAT-1**. COACH provides a central Secretariat to the **SEURAT-1** Research Initiative and to the SEP. Support to the cluster is provided either directly through the Scientific Secretariat or through the SEP.

An example of the scientific management and coordination is the development of a roadmap for the cluster as a whole: key contributions of the research projects, which are essential to meeting the above-mentioned cluster level objectives, were identified as the starting point

and introduced in the second volume of this book series. They were used to define the cluster-level milestones, and cross-cluster working groups were established and populated with delegates from the different project consortia. The working groups and the **SEURAT-1** projects need to interact with each other in order to achieve the three proof-of-concept levels, which form the backbone for the **SEURAT-1** roadmap (*Figure 1.1*). The overall approach for the implementation of this roadmap was developed by the coordination action project COACH in close cooperation with the project coordinators, and was subsequently endorsed by the SEP.

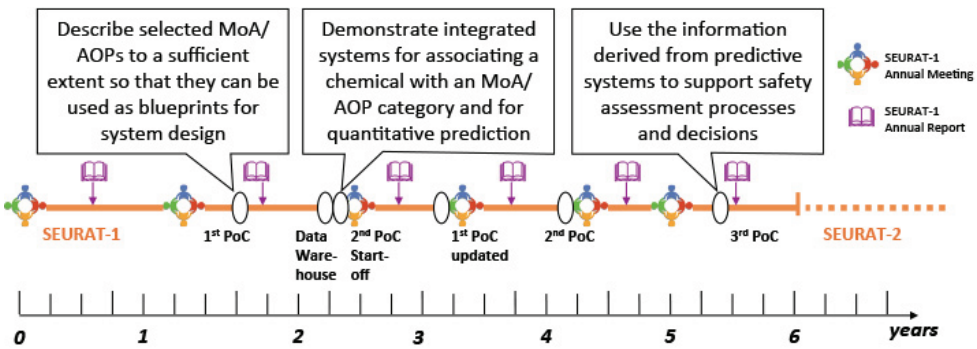


Figure 1.1 SEURAT-1 *roadmap illustrating the timing of the proof of concept (PoC) at three conceptual levels as the backbone for interactions between the* ***SEURAT-1*** *projects.*

The Annual Report: Something about 'Pathways'

This is the third volume of a series of six Annual Reports. The first volume presented a comprehensive overview of the planned work in the different projects of the **SEURAT-1** Research Initiative. The second and third volumes focus on highlights from the work periods in the research projects and steps towards reaching the final goal of the cluster. All six volumes together will provide a complete overview about recent cutting-edge research 'towards the replacement of *in vivo* repeated dose systemic toxicity testing' and, thus, represent a 'pathway' regarding scientific progress.

This leads to the common theme running through the Annual Report as well as through the **SEURAT-1** Research Initiative, as introduced in the first volume: the structure of the Annual Report, which will be kept over the six-year period, is inspired by one of the most important keywords of the addressed field of research, which is 'toxicity pathways' (*Figure 1.2*).

Briefly, chapter 2 describes developments in the legislative, regulatory and scientific contexts of the **SEURAT-1** Research Initiative. Chapter 3 outlines progress in the development of the long-term research strategy of the SEURAT initiative (i.e. **SEURAT-1** and beyond); in this third volume it presents the implementation of the research strategy through the selection of

toxicological modes-of-action that will be used to define the array of test systems needed to fulfil the requirements for safety assessment without animal testing. Overall, this will follow a case study approach and the design of such case studies is also outlined in chapter 3. This chapter is followed by detailed project descriptions in chapter 4, which provides an overview of research highlights in the past year. Finally, chapter 5 will focuses on related International Activities and identifies potential interfaces in order to establish collaborations for future research and development work leading to pathway-based human safety assessments in the field of repeated dose systemic toxicity testing of chemicals.

Conceptual considerations related to biological pathways leading to toxicity will consistently guide the report series. As a result, all six volumes together will show the pathway explaining how to perform the paradigm shift from describing phenomena to understanding processes in repeated dose toxicity.

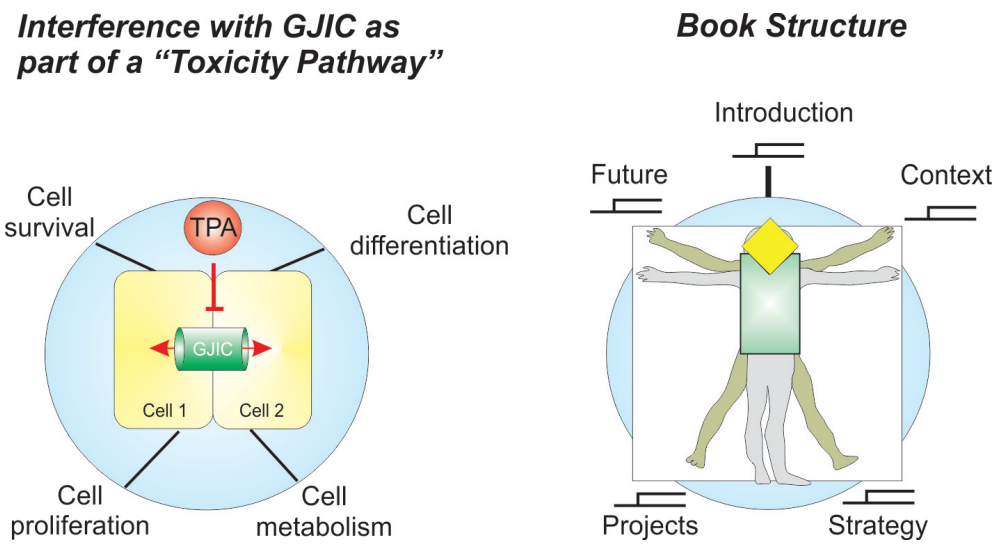


Figure 1.2 *The concept of 'Toxicity Pathways' (left panel) is mirrored by the book structure (right panel). Toxicity pathways may include cell-cell-interactions. Here we exemplify this process (by showing the effect of a chemical on gap junctional intercellular communication (GJIC, left panel).*

The Consortium and the Scientific Expert Panel (SEP)

The **SEURAT-1** Research Initiative combines the research efforts of over 70 European universities, public research institutes and companies. The composition is unique, as toxicologists, biologists from different disciplines, pharmacologists, chemists, bioinformaticians

and leading experts from other domains work closely together on common scientific objectives. The participation of SMEs in **SEURAT-1** is high, at more than 30%.

As described above, the Scientific Expert Panel (SEP) will advise the cluster on scientific matters related to specific topics within the area of repeated dose systemic toxicity. The SEP is composed of the project coordinators and external experts (the composition has changed slightly over the past year). The current membership is listed in *Table 1.1*.

Table 1.1 *Members of the* ***SEURAT-1*** *Scientific Expert Panel (co-leaders are highlighted in bold).*

Participant	Institution	Project
Project Coordinators		
Marc Peschanski	INSERM/UEVE 861, I-STEM/AFM, Evry / France	SCR&Tox
Mark Cronin	School of Pharmacy and Chemistry, Liverpool John Moores University / UK	COSMOS
Catherine Verfaillie	Interdepartmental Stem Cell Institute, Katholieke Universiteit Leuven / Belgium	HeMiBio
Jürgen Hescheler	Institute for Neurophysiology, University Hospital Cologne / Germany	DETECTIVE
Elmar Heinzle	Biochemical Engineering, Saarland University, Saarbrücken / Germany	NOTOX
Barry Hardy	Douglas Connect, Zeiningen / Switzerland	ToxBank
External Experts		
Hans Juergen Ahr	Bayer Health Care AG, Wuppertal / Germany	
Ian Cotgreave	AstraZeneca Safety Assessment, Södertälje / Sweden	
Catherine Mahony	Cosmetics Europe (Procter & Gamble), London Innovation Centre / UK	
Derek Knight	European Chemicals Agency, Helsinki / Finland	
George Daston	Procter & Gamble, Product Safety and Regulatory Affairs, Cincinnati / USA	
Russell Thomas	National Center for Computational Toxicology, U.S. Environmental Protection Agency, Research Triangle Park / USA	

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http://ec.europa.eu/research/horizon2020/index_en.cfm?pg=h2020-documents (accessed 26 April 2013).

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http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/animal_testing/com_at_2013_en.pdf (accessed 26 April 2013)

2 CONTEXT

'Today's entry into force of the full marketing ban gives an important signal on the value that Europe attaches to animal welfare. The Commission is committed to continue supporting the development of alternative methods and to engage with third countries to follow our European approach. This is a great opportunity for Europe to set an example of responsible innovation in cosmetics without any compromise on consumer safety.'

Tonio Borg, European Commissioner in charge of Health & Consumer Policy, 11 March 2013[1]

'The cosmetics industry is a flagship for Europe, which needs a strategy for growth and innovation; this needs to include a continuing commitment to encouraging alternatives development. Unfortunately, by implementing the ban at this time, the European Union is jeopardising the industry's ability to innovate.'

Bertil Heerink, Director General of Cosmetics Europe, 11 March 2013[2]

1-Press Release, European Commission (http://europa.eu/rapid/press-release_IP-13-210_en.htm, accessed 10 June 2013

2-Press Release, Cosmetics Europe (https://cosmeticseurope.eu/news-a-events/news/; accessed 10 June 2013)

2.1 Introduction

The 7th Amendment to the Cosmetics Directive introduced a number of key requirements related to animal testing, which were incorporated into the Cosmetics Regulation (Regulation (EC) No 1223/2009, 30 November 2009). In 2004 a ban was introduced on the testing of cosmetic products on animals within the EU. In 2009 an EU testing ban for cosmetic ingredients came into force with an extension of three specific areas: repeated dose toxicity (includes skin sensitisation, carcinogenicity and sub-acute/subchronic toxicity), reproductive toxicity (also includes teratogenicity) and toxicokinetics. On 11 March 2013 the full ban on animal testing for cosmetic products came into force and animal testing for marketing of new cosmetic products in the European Union was prohibited from this date.

In the first and second Annual Reports, the status of repeated dose toxicity testing in the context of safety assessment for cosmetic ingredients was described and using non-standard data (i.e., acquired from methods without the use of animals) for regulatory purposes was discussed. This chapter is intended to outline the recent developments in the legal, regulatory and scientific contexts of the **SEURAT-1** Research Initiative. Firstly, it highlights the consequences of the full implementation of the testing ban for cosmetic ingredients that came into force this year. Secondly, it outlines a framework for using currently available *in vitro* data to evaluate chemicals for safety. The field of safety assessment is the meeting point between regulators and scientists, and this section discusses an approach from a scientific perspective that could be implemented today. The following section places emphasis on the importance of biokinetics. Without quantitative understanding of the distribution of a test chemical in an *in vitro* system and without appropriate methods to extrapolate *in vitro* concentrations to the *in vivo* situation, *in vitro* based test systems will never find regulatory acceptance. Since much of the research within the **SEURAT-1** Research Initiative targets the development of *in vitro* methods for repeated dose toxicity testing, this section includes also some practical considerations for planning an *in vitro* experiment addressing repeated dose systemic toxicity. The last section of this chapter focuses then on the use of databases for extracting mechanistic information about toxicological modes-of-action with special emphasis on the molecular initiating event. Having in mind that the strategic orientation of the **SEURAT-1** Research Initiative is to adopt a adverse outcome pathway framework (as further discussed in chapter 3), knowledge about the starting point, which is the interaction(s) of a chemical with (a) molecular biological target(s), is essential.

2.2 Impact of the 2013 Animal Testing Ban

Rob Taalman

2.2.1 Setting the Regulatory Context

On 15 January 2003, the EU passed a law banning the testing of cosmetics and their ingredients on animals. Known as the 7th Amendment (*Anonymous, 2003*) to the Cosmetics Directive (*Anonymous, 1976*) it imposed a timeline for phasing out animal testing:

- Animal testing of finished cosmetic products in the European Union (EU) has been prohibited since 2004.
- Animal testing of cosmetic ingredients in the EU has been prohibited since 2009.
- From 11 March 2009, the marketing of cosmetic products and their ingredients that have been tested on animals is prohibited in the EU. This is to meet the requirements of the Directive. However, to ensure the continued safety of cosmetic products, the marketing ban allowed animal testing for only the most complex human health effects ('endpoints') (i.e., testing was permitted for repeated dose systemic toxicity, skin sensitisation, carcinogenicity, reproductive toxicity and toxicokinetics). The European Parliament and the Council extended this deadline until 11 March 2013.
- As a result, 11 March 2013 marked the completion of the implementation of the legislation and the instigation of the full EU marketing ban on products with ingredients tested on animals after March 2013 (*European Commission, 2013a*).

The Cosmetics Regulation, which replaces the Cosmetics Directive as of 11 July 2013, retains exactly the same provisions. Data from animal testing carried out before the respective implementation dates of the marketing ban (11 March 2009/11 March 2013) is permissible in the safety assessment of cosmetic products. However, the testing and marketing bans in the Cosmetics Directive/Regulation apply even where alternative methods to animal testing are not yet available.

This reflects a sector-specific political choice by the European Parliament and the Council. Other Union legislation recognises that animal testing is still required in the absence of alternative methods to ensure the protection of human health and the environment. This is coupled with very high animal welfare standards for such testing and demands that, whenever possible, animal testing is replaced, reduced and refined (*Anonymous, 2010*).

2.2.2 Availability of Alternative Methods

In Article 4a (2.3) of the Cosmetics Directive (*Anonymous, 1976*), the Commission was obliged to update the European Parliament and the Council on the status of replacement methods for animal safety testing for the purpose of complying with the Directive. The intention was for the Commission to check two years prior to the implementation of the marketing ban whether, for technical reasons, one or more of the tests covered by the 2013 marketing ban would not be developed and validated in time. Such an assessment would form the basis for considerations for advancing a legislative proposal to amend the regulation.

The Commission provided such a report in September 2011, based on a comprehensive technical report (*Adler et al., 2011*), built on the results of wide scientific input and public consultation. The conclusions of this technical report remain valid, with the full replacement of the 2013 marketing ban endpoints by alternative methods still not yet possible. However, the report recognised that considerable progress has been made in recent years; for the endpoints falling under the 2009 marketing ban, replacement methods have been successfully validated and adopted as OECD test guidelines for skin irritation and corrosion, phototoxicity and skin penetration. Partial replacement methods suitable for inclusion in testing strategies have also been validated for acute systemic toxicity and eye irritation, and adopted as OECD test guidelines for eye irritation. Refinement of the established *in vitro* genotoxicity tests and testing strategies will contribute to higher specificity of the test systems and therefore more reliable predictions for this endpoint.

For the 2013 marketing ban endpoints, some test methods for skin sensitisation and carcinogenicity are now being discussed at the OECD. A summary of available OECD-approved methods relevant for the safety assessment of cosmetic products can be found in *Table 2.1*. For the remaining complex endpoints, adequate replacement will not be achieved by replacing one animal test with one *in vitro* test. Replacement requires integrated testing strategies that combine various approaches. For example, none of the methods referenced in *Table 2.1* currently being validated will replace existing skin sensitisation testing as a stand-alone method; they constitute a mosaic of pieces needed for a comprehensive testing strategy. In addition, while current methods do provide information on hazards they are insufficiently developed for risk assessment purposes.

Table 2.1 *Internationally Accepted Alternative Test Methods for Cosmetic Products/Ingredients Safety Testing (based on European Commission, 2013b).*

Method	International Acceptance
Dermal Corrosivity Test Methods	
CORROSITEX Skin Corrosivity Test	OECD TG 435 (2006)
EpiSkin Skin Corrosivity Test	OECD TG 431 (2004)
EpiDerm Skin Corrosivity Test	OECD TG 431 (2004)
SkinEthic RHE Skin Corrosivity Test	OECD TG 431 (2004)
EST-1000 Skin Corrosivity Test	OECD TG 431 (2004)
Rat TER Skin Corrosivity Test	OECD TG 430 (2004)
Dermal Irritation Test Methods	
In Vitro reconstructed human epidermis (RhE) test methods EpiDerm EPISKIN SkinEthic	OECD TG 439 (2010)
Phototoxicity Test Methods	
3T3 NRU Phototoxicity Test	OECD TG 432 (2004)
3T3 NRU Phototoxicity Test: Application to UV Filter Chemicals	OECD TG 432 (2004)
Ocular Toxicity Test Methods	
Bovine Corneal Opacity and Permeability (BCOP) Test Method for identification of ocular corrosives and severe irritants	OECD TG 437 (2009)
Isolated Chicken Eye (ICE) Test Method	OECD TG 438 (2009)
Fluorescein Leakage Test Method for Identifying Ocular Corrosives and Severe Irritants	OECD TG 460 (2012)
Use of histopathology as an additional endpoint in Ocular safety testing	OECD Guidance Document 160 (2011)
Immunotoxicity (Allergic Contact Dermatitis) Test Methods	
Murine local lymph node assay (LLNA) for skin sensitization	OECG TG 429 (2002) ISO (2002)
Update Murine local lymph node assay (LLNA) for skin sensitization (20% reduction)	Update to TG 429 OECD (2010) ISO (2010)
Reduced LLNA (rLLNA)	Update to TG 429 (2010) ISO (2010)

Nonradioactive LLNA protocol (LLNA: BrdU-ELISA)	OECD TG 442B (2010)
Nonradioactive LLNA protocol (LLNA:DA)	OECD TG 442A (2010)
Harmonized Performance Standards for the LLNA	Update to TG 429 (2010)
Acute Toxicity Tests	
Up and Down Procedure (UDP)	OECD TG 425 (2008)
Fixed Dose Procedure (FDP)	OECD TG 420 (2001)
Acute Toxic Class Method (ATC)	OECD TG 423 (2001)
Acute inhalation toxicity	OECD TG 403 (2009)
Inhalation toxicity – acute toxic class method	OECD TG 436 (2009)
In vitro cytotoxicity test methods for estimating starting doses for acute oral systemic toxicity tests	OECD Guidance document 129 (2010)
Endocrine Disruptor Test Methods	
Performance-Based Test for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists	OECD TG 455 (2012)
BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists	OECD TG 457 (2012)
Genetic Toxicity Test Methods	
In vitro micronucleus Test	OECD TG 487 adopted (2010)

2.2.3 Assessing the Impacts of the 2013 Marketing Ban

All major EU policy initiatives and legislative proposals considered by the European Commission need to undergo an impact assessment. This follows an integrated approach introduced in 2002; it replaces the previous single-sector type assessments and examines the potential impacts of new legislation or policy proposals in economic (including competitiveness), social and environmental fields. It is underpinned by the principle of proportionate analysis, whereby the depth and scope, and hence the resources allocated to it, are proportionate to the expected nature of the proposal and its likely impacts. Wide-ranging consultation with stakeholders is an integral part of the approach.

The Commission's integrated impact assessment therefore took into account the fact that a full set of alternative methods (see above) was not yet available. The results were published on 11 March 2013 as a Commission staff working document accompanying the Communication from the Commission on the animal testing and marketing ban.

The options assessed were to:

- maintain the 2013 marketing ban;
- postpone it;
- introduce a derogation mechanism.

A derogation mechanism would have allowed manufacturers to request the Commission to grant individual derogations from the 2013 marketing ban for innovative ingredients. This would have offered significant benefits for consumer health, well-being and/or the environment.

The assessment showed that implementing the 2013 marketing ban could reduce access to cosmetic ingredients. There was also the risk that it would have a negative effect on innovation in Europe, possibly leading to relocation of research and development (R & D) outside the EU. However, the Commission stated that despite serious efforts to establish a solid body of data, it was impossible to quantify these impacts with any certainty. This is an on-going dilemma; by definition, impact assessments are difficult to interpret. 'Data' quality is always an issue, since inevitably these are largely predicted/projected values rather than measured and actual. The Commission concluded that there may be a socioeconomic impact of the ban but due to uncertainty of the scope of this impact, it has limited value and as a result would not be taken into account. Ironically, in taking such a stance the EC is highlighting the shortcomings of its own approach. This has been seemingly belatedly recognised, with the EC now considering reviewing its impact assessment guidelines to increase its validity as a decision-making tool.

2.2.4 The Way Forward - *A* Political Decision

In light of the conclusions of the impact assessment, the Commission took the decision to allow the 2013 marketing ban to enter into force without any legal proposal to either postpone the deadline or provide for individual derogations. Amongst the reasons they listed were:

- Firstly, it believes that any further postponements of the 2013 marketing ban would not be in the spirit of the political choices of the European Parliament and the Council when adopting the respective provision.

  From the outset, animal welfare was the major driver for agreement on the first provisions of the marketing ban. First introduced in 1993 with a deadline of 1998, the marketing ban had the clear political objective of ending animal testing for cosmetics. The technical feasibility of a full set of alternative methods becoming available was not a consideration. Similarly, the European Parliament and the Council imposed both the testing ban and the 2009 marketing ban in the full knowledge that a complete replacement of the relevant animal tests would not be possible within the available timeframe. They did not make the 2013 marketing ban dependent on the availability of a full set of replacement methods. In reality, the provision in the regulation to perform a technical feasibility study two years prior to the implementation of the 2013 ban was apparently 'for information only' and without further consequences. This appears at odds with the EU's stated desire to take informed, knowledge-based decisions.

- Secondly, the Commission feels that any change to the 2013 marketing

ban could seriously undermine motivation to develop further alternative test methods. It argues that past experience demonstrates that removing animal testing provisions from cosmetics legislation has been a key accelerator in relation to the development of alternative methods and has increased awareness and support for the possibilities of animal-free safety testing far beyond the cosmetics sector and far beyond Europe.

It is correct that methods developed in the cosmetics sector, such as reconstructed human skin models, are now being used in other sectors, and the interest in alternative methods for cosmetics has increased in a number of countries outside the EU. However, there is no evidence that the ban will accelerate development and acceptance of alternatives. More importantly, if the EU wishes to have a high-profile role in this field, proper incentives should be offered to stimulate R & D investments. Without these, it is possible that the private sector may favour investing in regions with growing markets in preference to somewhere where innovation is difficult or impossible.

➠ The animal testing provisions for cosmetics were a motivating factor for the creation of the European Partnership on Alternative Approaches to Animal Testing (EPAA). This is a unique collaboration between the European Commission, European trade associations, and companies from various industry sectors. The partnership promotes the concepts of 3'R's (replacement, refinement and reduction of animal use) in all areas of safety testing including food, pharmaceuticals, vaccines and chemicals. While it is important to develop alternative methods, it is of even greater importance to get those methods accepted by regulators. This is an issue not only for one-to-one replacement methods but also for those methods that can only be informative in combination with other techniques. This requires a shift in mindset from regulators and a better understanding of the predictive value for potential effects (short- and long-term) in humans.

➠ Finally, it considers that the potential risks from the 2013 marketing ban are outweighed by the opportunity for the EU to set an example of responsible innovation in cosmetics with benefits beyond Europe. The need for a new risk assessment paradigm from a scientific perspective is widely recognised.

The Commission's ambition should be applauded; it is in the interests of all stakeholders to have access to the best possible tools and approaches that guarantee consumer and environmental safety. This can only happen if science is given the time and resources required for delivering the methods and when regulators are convinced that the alternative methods increase public confidence in safety assessment and thereby in risk management.

The expectation seems to be that the cosmetics sector can – once again – act as an accelerator and a pioneer in the development of tools that are cheaper, quicker, better and more predictive in assessing consumer safety. However, given that a full safety assessment for cosmetics relying only on alternative methods and approaches has not yet been achieved and in some respects is not even close, the Commission has committed to certain actions including:

- monitoring the effects of implementing the 2013 marketing ban;
- continuing to support research, development and validation of alternative methods to assess human safety;
- making alternative methods part of the Union's trade and international agenda.

It remains to be seen whether this politically motivated 'framework' will be effective in maintaining the EU research efforts at their current levels.

2.2.5 The Importance of Research and the SEURAT-1 Research Initiative

The **SEURAT-1** Research Initiative is an example of the active role the cosmetics industry has adopted in developing alternative testing methods. The goal of the five-year work programme of the **SEURAT-1** Research Initiative is to use knowledge of toxicological processes to develop and rationally assemble novel 'alternative' tools that can predict repeated dose systemic toxicity in humans, potentially caused by exposure to chemicals. Ultimately, **SEURAT-1** aims to prove key concepts underpinning the credible use of combinations of *in silico* and *in vitro* methods for supporting safety assessment decisions.

Research into alternative methods is by no means nearing an end; in many areas the current research is only the first step. Once the **SEURAT-1** Research Initiative ends in 2015, further research will be required. It seems logical that future work should be financed via Horizon 2020. If the EU wishes to maintain its leading role in this field, Horizon 2020 should give top priority to developing alternative, better methods for human safety assessment.

It is important to set future research priorities, along with the best implementation instruments. These could include a new public-private partnership. A recent Discussion Paper of the Scientific Committees entitled 'Addressing the New Challenges for Risk Assessment' points to research needs for comprehensive open access databases, *in silico* methods, (toxicological) mode-of-action studies and exposure assessment tools (*Scientific Committees, 2012*).

The EPAA also has a role to play in shaping research needs and priorities across industry sectors, with a focus on how best to involve small- and medium-sized enterprises. A critical success factor will be ensuring that alternative methods, once developed, are made available to end-users as quickly as possible, so that they can generate toxicological information that is acceptable to regulators.

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2.3 A Data-Driven Framework for Incorporating New Technologies into Chemical Safety Assessment

Russell S. Thomas

Introductory remark: This is an extended abstract of a manuscript that was submitted to Toxicological Sciences.

2.3.1 Introduction

The release of the US National Research Council's Report 'Toxicity Testing in the 21st Century: A Vision and a Strategy' in 2007 initiated a broad-based movement in the toxicology community to re-think how toxicity testing and risk assessment are performed. Since the release of the report, multiple efforts in the US and abroad have added to the momentum, but the majority of these efforts have focused more on a vision of how things should be done rather than the development of a data-driven paradigm. The focus on vision has been due to a lack of adequate datasets that would be required. This lack of data is gradually changing with the release of the ToxCast Phase I data, the development of high-throughput dosimetry approaches, and the collection of short-term *in vivo* transcriptomic studies.

2.3.2 Selective versus Non-selective Chemicals

Over the past five years, a series of studies has been conducted that together may begin to formulate a new data-driven framework to toxicity testing and risk assessment (*Figure 2.1*). The first study is a comprehensive cross-validation model comparison to evaluate the predictive performance of the more than 600 *in vitro* assays from the ToxCast Phase I screening effort across 60 *in vivo* endpoints using 84 different statistical classification methods. The predictive performance of the *in vitro* assays was compared to that of chemical structure descriptors. The results showed that the current suite of ToxCast high-throughput toxicity assays have limited applicability for predicting *in vivo* chemical hazards using standard statistical classification methods (*Thomas et al., 2012a*). Despite these limitations, the *in vitro* assays do provide a broad survey of the potential proximal biochemical and cellular targets for a chemical, and can be used to separate chemicals into either those that cause toxicity primarily through non-selective interactions with cells and cellular macromolecules or those that act through more selective interactions (e.g., receptor-mediated chemicals). When analysed from this perspective, the data suggest that the majority of chemicals represented in the ToxCast Phase I library likely act via non-selective interactions with cellular macromolecules.

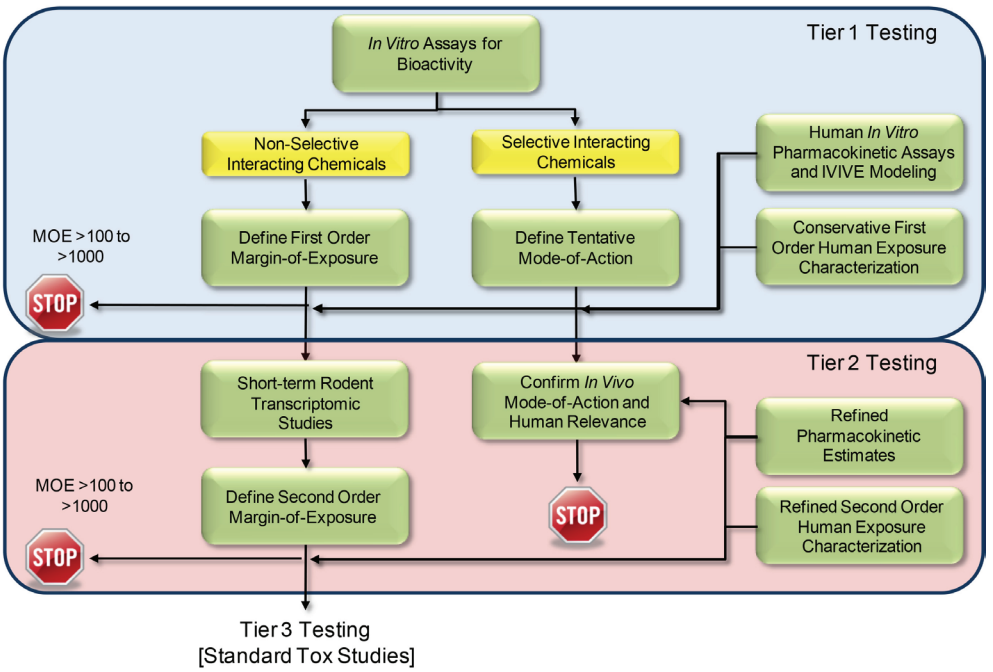

Figure 2.1 *Flow-chart outlining a proposal for a data-driven framework to toxicity testing and risk assessment.*

The internal dose of a chemical is an important determinant of toxicity. In previous studies, data from *in vitro* hepatic metabolic clearance and plasma protein binding assays were used to parameterise an *in vitro*-to-*in vivo* extrapolation (IVIVE) model to estimate the daily human oral dose, called the oral equivalent dose, necessary to produce steady-state *in vivo* blood concentrations equivalent to the *in vitro* AC_{50} value for each of the high-throughput *in vitro* assays (*Rotroff et al., 2010; Wetmore et al., 2012; 2013*).

For the selective chemicals, the oral equivalent dose values could be used to identify potential key events in a mode-of-action (MoA). The MoA for a chemical consists of a series of key events triggered by a chemical at a molecular or cellular level that causally link with an *in vivo* adverse effect. Therefore, the key event must be triggered at doses lower than or equal to doses at which the adverse effect is observed. Among a subset of ToxCast Phase I chemicals, eight were active in the *in vitro* peroxisome proliferator-activated receptor alpha (PPARa) assays and caused rat proliferative lesions, while four also caused rat liver tumours. For each chemical, the lowest observed effect level (LOEL) and no observed effect level (NOEL) for rat liver proliferative lesions and tumours were overlaid with the oral equivalent dose values for the three high-throughput *in vitro* assays measuring PPARa-related activation (*Wetmore et al., 2013*). In all cases, activation of the PPARa *in vitro* assays occurred at or below the dose

for the *in vivo* effects, thereby demonstrating consistency as potential key events on a dose level. From these selectively activated or inhibited assays, probable key event(s) could be determined and the oral equivalent dose value for the key event could be used as the point-of-departure (PoD) in the dose-response assessment.

For the non-selective chemicals, identifying the MoA is neither efficient nor economical since by definition, the chemical will interact with and perturb multiple cellular processes within a narrow dose range. In a previous study, rat-specific oral equivalent doses were calculated for 59 ToxCast Phase I chemicals and compared with LOEL values for a variety of *in vivo* apical responses in the rat (*Wetmore et al., 2013*). Although not significantly correlated, the oral equivalent dose for the most sensitive *in vitro* assay was less than the LOEL for the most sensitive rat *in vivo* endpoint for approximately 95% of the chemicals. On average, the most sensitive *in vitro* assay was 66-fold lower than the most sensitive LOEL value. These results demonstrate that the oral equivalent dose for the most sensitive *in vitro* assay can be used as a conservative estimate of the PoD for the non-selective chemicals in the dose-response assessment.

Estimates of human exposure would be combined with the PoD from the dose-response assessment to calculate a margin-of-exposure (MoE). For the selective chemicals, the MoE could be calculated relative to the oral equivalent dose from the *in vitro* assay(s) associated with the proposed key event. For the non-selective chemicals, the MoE could be calculated relative to the oral equivalent dose for the most sensitive *in vitro* assay. For those chemicals with an MoE greater than a defined cut-off, no further testing would be required while chemicals with an MoE below the cut-off would progress on to the next tier (*Figure 2.1*). In a previous study on the ToxCast Phase I chemicals, an MOE cut-off of >100 would eliminate additional testing on ~40% of chemicals while a MOE cut-off of >1,000 would eliminate additional testing on ~25% of chemicals (*Wetmore et al., 2013*).

For chemicals progressing on to the next tier, data requirements would be different for the selective and non-selective chemicals. For the selective chemicals, focused *in vivo* MoA studies would be performed to confirm the tentative MoA defined in the first tier and would be defined by the type of MoA proposed. Assuming the MoA is relevant to humans, the PoD would be determined based on the associated endpoints in the *in vivo* studies.

For non-selective chemicals, a series of previous studies have examined the relationship between transcriptional and apical responses (*Thomas et al., 2007; 2011; 2012b; 2013*). Both non-cancer and cancer-related responses were analysed using benchmark dose (BMD) methods to identify PoDs. The dose-response changes in gene expression were also analysed using BMD methods and the responses grouped based on signalling pathways. A comparison of transcriptional BMD values for the most sensitive pathway with BMD values for the non-cancer and cancer apical endpoints showed a high degree of correlation (*Thomas et al., 2012b*). The correlation was also robust across multiple time points (*Thomas et al., 2013*).

Dose-response studies could be performed on the non-selective chemicals at any single time point between 5 days and 13 weeks. The studies would be performed in mice and rats of both sexes. A battery of eight tissues that include those most frequently positive in rodent cancer bioassays (liver, lung, mammary gland, stomach, vascular system, kidney, hematopoietic system and urinary bladder) would be collected. These eight tissues cover 92% and 82% of targets for all mouse and rat carcinogens, respectively (*Gold et al., 2001*). Gene expression microarray analysis on these tissues would allow the estimation of pathway transcriptional BMD and BMDL values. The signalling pathway with the lowest transcriptional BMD value across all analysed tissues would be used to derive the PoD.

Similar to the initial tier, refined estimates of human exposure would be combined with the PoD from the dose-response assessment to calculate an MoE. For the selective chemicals, the MoE could be calculated relative to the PoD of the associated endpoints in the *in vivo* studies. For the non-selective chemicals, the MoE could be calculated relative to the PoD derived from the signalling pathway with the lowest transcriptional BMD value across all analysed tissues. For those chemicals with an MoE greater than a defined cut-off, no further testing would be required while chemicals with an MoE below the cut-off would progress on to the final tier (*Figure 2.1*). Using data from ToxRefDB on the *in vivo* LOEL values for the ToxCast Phase I chemicals and the corresponding human exposure estimates, MoE values were calculated using the minimum *in vivo* LOEL value (i.e., the most sensitive *in vivo* endpoint) and the exposure estimate for the most highly exposed subpopulation. Assuming that this ratio is representative of the MoE values for a larger set of chemicals, an MoE cut-off of >100 would eliminate additional testing on ~97% of chemicals while a MoE cut-off of >1,000 would eliminate testing on ~85% of chemicals.

The studies proposed for 3–15% of chemicals passing into the final tier are not explicitly defined, but they would be conceptually equivalent to the current guideline *in vivo* studies performed on high-value chemicals with significant potential for human exposure. These studies could include rodent cancer bioassays, developmental toxicity studies and two-generation reproductive toxicity studies. Depending on the MoE cut-off values imposed, the vast majority of chemicals would be screened out in the preceding tiers.

2.3.3 Conclusions

The proposed tiered testing framework is an attempt to integrate data from new technologies into toxicity testing using the best science available at this point in time. The framework is a significant departure from the current way we evaluate and test chemicals for toxicity and will require a shift in thinking about chemical safety. The first shift revolves around the separation of chemicals into selective and non-selective mechanisms and the acceptance that the majority of non-pharmaceutical chemicals act via non-selective means. The prevailing thought is that the MoA and adverse outcome pathway (AOP) approach can be efficiently applied to

all chemicals; however, imposing an MoA or AOP approach on non-selective chemicals would waste valuable resources and unnecessarily delay decision-making since each MoA/AOP requires agreement on the underlying key events followed by extensive peer review. These efforts should be focused on the selective chemicals where a series of standardised MoAs could be developed that are associated with specific cellular targets.

A second shift in thinking will be required for the non-selective chemicals. The current hazard-based labelling approach that relies on apical responses will need to be transitioned to a 'region of safety' approach where the most sensitive adverse apical effect is not known, but the PoD and subsequent decisions are based on the lack of biological perturbation. This shift in thinking may be more challenging to overcome due to (i) the entrenched reliance on apical responses and concerns relating to the broad applicability of these associations; (ii) the potentially conservative nature of relying on molecular perturbations as PoDs; and (iii) the difficulty of associating molecular perturbations with actual risk.

In summary, the proposed framework provides a risk-based, rapid, cost-effective and animal-sparing means to evaluate chemicals for safety. The framework represents only an initial step along the way to a fully knowledge-based approach for evaluating chemical safety, but progress towards a better future in chemical safety assessment will require implementation of pragmatic approaches, such as the proposed framework or one similar to it, by national regulatory agencies, cooperation of international organisations, and stakeholder education and involvement.

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2.4 The (Sometimes Missing) Link: Biokinetics

2.4.1 The Importance of *in vitro* Biokinetics

Bas Blaauboer

In vitro assays have different applications in toxicology, one of these being the search for the key events occurring after cells are exposed to a chemical substance, in order to elucidate the primary mechanism of action. Another application is the use of a cell-based assay in screening for a particular cellular endpoint of toxicity. The result of such a study is often the quantification of the effect, either as an EC50 (i.e., the concentration at which 50% of the maximal effect occurs), as the LOEL (lowest observed effect level) or something similar (e.g., benchmark dose values). When these data are to be used in evaluating the toxicity profile of the chemical in an intact organism, the *in vitro* data will have to be evaluated in the context of the *in vivo* situation, a process referred to as (quantitative) *in vitro–in vivo* extrapolation (QIVIVE) (*Blaauboer, 2010; Yoon et al., 2012*).

An important issue in all these *in vitro* approaches is a proper quantification of the exposure to the chemical: at the cellular level or, even more preferred, at the level of the toxicological target. This is of particular interest when a QIVIVE has to be performed, especially when there are reasons to assume that the biokinetic behaviour of the chemical will differ in the *in vitro* system as compared to the *in vivo* situation.

Factors that can contribute to these differences are: binding to proteins, if there are considerable differences in protein content of medium versus blood plasma; evaporation; and binding to the plastic component of the culture system (*Gülden et al., 2002; Kramer et al., 2012*). This might lead to differences in cellular or target exposure. An approach to overcome these differences is the measuring or modelling of the freely available concentration in the medium surrounding the cells, in comparison to this concentration in blood plasma (*Kramer et al., 2009*). Another important factor is the fact that many *in vitro* systems lack metabolic capacity or show strongly reduced metabolism in comparison to *in vivo* (*Coecke et al., 2006*). Moreover, if metabolism occurs in a stationary culture metabolites are not cleared and remain in the culture until the medium changes. Other differences between *in vitro* and *in vivo* conditions include the cell density, cell proliferation and the stability of the system during the entire period of incubation.

All these factors will influence the concentration of the compound (or its active metabolite) that the target actually 'sees'. Therefore, the biokinetic behaviour of the chemical should be an integral part of the study protocol for any *in vitro* system used in toxicology studies. Knowledge of this '*in vitro* biokinetics' is essential not only for the later *in vitro–in vivo* extrapolation (QIVIVE), but it also delivers the essential parameters required to optimise the application schedule (selection of dose and time interval of application) for the repeat dose *in vitro* experiment.

In connection with this, consideration should be given to the choice of the proper dosimetry in the *in vitro* system. Depending on the nature of the toxic interaction, the most relevant dosimetry can be described by the course of the concentration over time (i.e., the area under the curve), a peak concentration, or a concentration above a certain threshold. Thus, three elements – critical dosimetry, critical compound (viz., parent or metabolite) and critical site of action – form the basis of our understanding of the toxicity of a chemical, together with comprehending the physiological relevance of these interactions (*Blaauboer, 2010*).

Another issue is the interpretation of the *in vitro* toxicity data with regard to their relevance for the *in vivo* situation. Cellular systems lend themselves to measuring a range of effects and in this context it is sometimes difficult to select the relevant biomarker of toxicity for further consideration of the compound's toxicity pattern (*Blaauboer et al, 2012*). And even if the relevant concentration–effect relationship in the *in vitro* system is identified, a distinction needs to be made between those changes that are clearly adverse and weaker effects that could be considered as being within the range of adaptive variations (*Figure 2.2*).

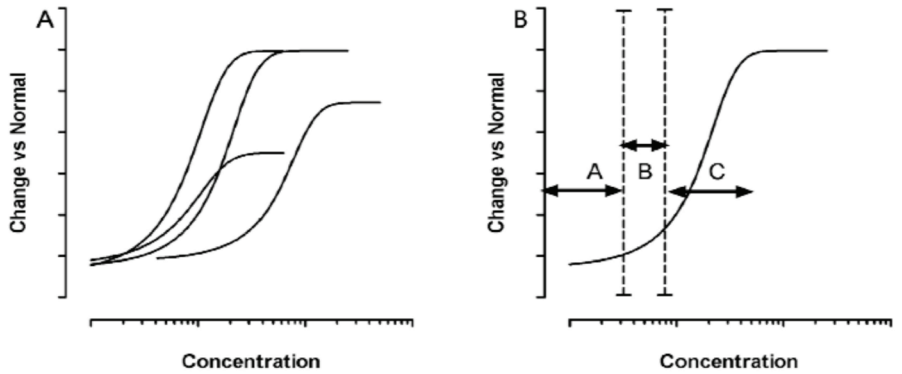

Figure 2.2 *Interpretation of concentration–effect relationships for in vitro experiments. (A) Examples for the many possible relationships between a compound's concentration and endpoint changes within one given experimental system. (B) Definition of concentration thresholds related to adversity (region A: compound concentration does not affect the chosen biomarker of toxicity; region B: significant change of the biomarker of toxicity from its baseline, but not predictive for adversity; region C: change of the biomarker of toxicity indicates adversity). Data source: Blaauboer et al. (2012)*

As shown in *Figure 2.2A*, different endpoints react at different compound concentrations. Some of these changes will be a reflection of adaptation or they may be unrelated to the eventual cell fate. Some will be related to adversity, or they will reflect a pathway of toxicity relevant for cell fate and for *in vivo* toxicity prediction. In addition, also the time factor will have an effect on the shape of the curves: duration of exposure, timing of (short-term) exposure within a more extended experimental protocol, and timing of measurement. As a consequence, choices will have to be made for selecting the most relevant of these endpoints as biomarkers of toxicity.

If a choice has been made for one or more of the relationships in *Figure 2.2A* to be used as biomarkers of toxicity, the next step is to define concentration thresholds related to adversity. For each biomarker of toxicity, ranges of compound concentrations can be observed that do not affect the biomarker (*Figure 2.2B*, region A). In other concentration ranges (*Figure 2.2B*, region B) the biomarker of toxicity changes significantly from its baseline, but this effect does not predict adversity. In a third concentration range (*Figure 2.2B*, region C), the change of the biomarker of toxicity is related to adversity.

In summary, the definition of biomarkers of toxicity as predictive tools needs to take into account both the dynamics and the kinetics of the compound in the *in vitro* systems.

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2.4.2 Short Guidance for the Development and Use of Cell Culture Systems Aiming to Predict Repeat Dose Systemic Toxicity

Michael Schwarz

Some general points

The following points have to be considered when a test system is intended to be used for prediction of a certain adverse effect (following general principles on test validity as outlined by *Hartung et al., 2004*):

➠ What is the purpose of the test method?

Describe in as much detail as possible what the test intends to predict. *Example:* The test will predict cholestasis induced by inhibitors of the bile salt export pump (BSEP).

➠ What is the mechanistic basis of the test method?

Describe in as much detail as possible the mechanistic basis of the test method. *Example:* The assay is able to measure BSEP activity with high accuracy. Inhibition of BSEP activity in hepatocytes is known to induce a cholestatic response *in vivo*. Therefore, the assay is able to predict this response to BSEP inhibitors. However, cholestasis can be induced by several mechanisms other than BSEP inhibition and these mechanisms are not covered by the assay.

➠ What is the predictive capacity of the test assay?

For evaluation of the predictive capacity and the definition of a (provisional) domain of applicability a set of positive and negative test compounds has to be evaluated. A prediction model has to be defined from the data and/or the procedure for data analysis of the training set has to be described.

➠ What is the relevance of the test method within a regulatory context?

Example: The assay can be part of a test battery or testing strategy aiming to predict cholestatic activity of test agents.

➠ What is the reproducibility of your test within your laboratory?

The standard operation procedures (SOP) of the test must be well defined and described in detail. Information on the reproducibility of the experimental data within the laboratory is obligatory.

Frequently Asked Questions in the context of repeated dose in vitro experiments

➠ How long do I need to incubate my cell culture system with test compounds to optimally simulate the *in vivo* situation?

This is one of the most frequently asked questions and, unfortunately, there is no general answer to this. The answer will depend on, for example, the particular test system in question and the readout that is used, just to mention two points. Certainly the answer ‘incubate your cells for 14 or 28 or 90 days, since this corresponds to the sub-acute and sub-chronic test schemes used in toxicity testing in the experimental animal’ is not a relevant one, for very obvious reasons related to differences in the biokinetic and toxicodynamic behaviour of test chemicals *in vitro* as compared to *in vivo*. Two examples may give some guidance.

Example 1: Let us assume we have a cell-based reporter system that as readout for toxicity employs the activity of the stress-activated transcription factor Nrf2. Let us further assume that the mode-of-action (MoA) of the test chemical is through production of reactive oxygen species (ROS). What we know is that ROS will trigger Nfr2 activation in an ROS concentration-dependent manner, which can be measured by quantification of the activity of the Nrf2-regulated reporter. Let us now assume that we expose our cells for several days to a slightly cytotoxic concentration of a test compound and keep the concentration constant over time. Within hours of the start of incubation activation of Nrf2, it will induce an adaptive transcriptional response increasing the expression of genes encoding ROS-detoxifying enzymes. The activity of the reporter readout will be proportional to the actual concentration of ROS in the cell system and, in the low concentration range, chronic exposure will not lead to an increase in toxicity as a function of time of incubation.

Example 2: Let us assume we have a second test agent that has protein-alkylating properties as an MoA. Protein alkylation may cause irreversible damage to protein function and repair is only possible by *de novo* protein synthesis, which will often be slow. Let us also assume we have a cell system with global gene expression measured by transcriptomics as a readout. What we expect to see is a stress response triggered a few hours after the start of incubation and this will be somehow proportional to the concentration of the chemical in the assay. However, because of slow damage repair we will now see accumulation of damage over time which may be indicated, for example, by a shift in the intermediary metabolism of cells towards glycolysis. With such a compound the degree of response may be related to the total dose (i.e., AUC) and not simply to actual concentration; exposure of cells to small concentrations for longer periods of time may produce a similar effect as a high concentration with a short incubation period ('c x t poison behaviour').

General recommendations: The optimal length of incubation will depend on the MoA of the agent tested and on the readout used. In very general terms, use of several concentrations AND several time points of analysis are recommended to allow an analysis of dose- or effect-additivity.

➠ How should I consider the proper dosimetry in the *in vitro* system?

The first consideration should be the most relevant target exposure scenario in the *in vivo* situation. One option is to select the condition that most closely mimics the exposure of target cells *in vivo*. This might mean the use of protein concentrations in the medium that are equal or close to the plasma protein concentration; however, some cellular systems might not allow this. In such cases the optimal solution would be to either measure or model the freely available concentration. In any case the biokinetic behaviour in the *in vitro* system should be considered.

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2.5 Chemical Structure–Based Toxicity Databases: Insight into Molecular Initiating Events

George P. Daston

The prediction of the toxic properties of chemicals has traditionally been assessed using whole animal, usually mammalian, models. These models are phylogenetically close to humans, with similar biology and often similar disease processes. Over the past 50 or more years, regulatory agencies have developed research protocols and guidelines for the conduct and interpretation of laboratory animal toxicity studies. Tens of thousands of studies have been conducted over this period of time, on thousands of chemical entities. These studies form the basis of most regulatory decisions on the classification and safe use levels of chemicals in commerce and the environment.

The data derived from these studies is adverse outcome data, and is almost always at a high level of biological organisation (i.e., the organ or organismal level). In some cases, it is possible based on information generated in mechanistic studies, or by using additional data gathered in guideline toxicology studies, to make inferences about the mode-of-action of a chemical. For example, mechanistic studies on the reproductive and developmental effects of anti-androgens have shown that selective antagonists of the androgen receptor (*McIntyre et al., 2001*), or inhibitors of the enzyme 5-alpha-reductase (*Clark et al., 1990*), given to pregnant rodents results in a characteristic pattern of morphological effects in male offspring, such as a reduction in anogenital distance, retention of nipples, and at high dose levels a low incidence of structural malformations of the reproductive system. If a newly tested chemical produces a comparable pattern of effects in a developmental toxicity study, it would be possible to infer that the new chemical acted by the same mode-of-action.

There is considerable value in using the existing toxicity data set to provide much greater insight into the potential toxicity of untested chemicals, both at the outcome level and at the mode-of-action level. Compounds with similar chemical features can often be expected to

have the same biological targets. This is the basis for molecular design in drug development, and is also the underlying foundation for read-across in regulation. Two challenges to the use of the historical data have been easy access to the data based on chemical structure, and chemistry rules for judging the likelihood that two chemicals share a biological target. Both of these challenges have been addressed.

The toxicology data that are available in the peer-reviewed and grey (unpublished, but available publicly as regulatory submissions) literature have been compiled by the US Environmental Protection Agency into a relational database that is searchable by two-dimensional chemical structure and substructure (*Richard et al., 2008*). The database, called DSSTox (for Distributed structure-searchable toxicity), contains entries for all toxicity endpoints. It is also well curated with excellent quality control to ensure that structures match names and CAS numbers. Queries can be submitted by drawing a chemical structure, by chemical name or CAS number, or by more advanced chemical notation methods. The search engine includes a program that evaluates the extent of similarity of the identified analogues to the chemical being queried, providing some guidance as to how useful the data on that particular analogue might be. A second database that has been compiled by the US Environmental Protection Agency is the Aggregated Computational Toxicology Resource (ACToR), which searches exposure, use and other information (e.g., ToxCast) along with hazard data for additional insight into the toxicity of a queried chemical (*Judson et al., 2012*). Together, these resources provide all toxicologists with the necessary tools to search the large body of toxicology literature for analogues of interest.

The second challenge is the codification of chemistry rules for judging the suitability of analogues, beyond what an overall assessment of chemical similarity might do. There are a number of recent publications that provide guidance in this area, focusing especially on reactivity, structural, metabolic and physical chemistry similarities (*Ellison et al., 2009; Wu et al., 2010*). These systems have been tested (e.g., *Blackburn et al., 2011*) with a series of case studies and found to be robust and consistent. Advances are also being made in the identification of chemical features necessary for binding to specific receptors, including estimates of relative affinity (*Schmieder et al., 2003*).

The next step in the use of large databases for predictive toxicology is to more closely connect chemical structure with molecular initating event. This will permit the use of the relatively new data streams of high-throughput (e.g., ToxCast) and high content (e.g., toxicogenomics) as supporting information in read-across. It will also provide considerable support for the development of adverse outcome pathways. It should be clear that the initial interaction of a chemical is with a (bio)chemical target within the organism. For example, the initial interaction of a mutagen with the biological system is with the nucleotide portion of DNA. The biologically significant interaction of an estrogen is with the binding domain of an estrogen receptor. These initial chemical events can then lead to effects at higher levels of biological organisation, if

there are enough of them. As we continue to develop an understanding of the relationship of chemical features with biological outcome, it is becoming possible to predict molecular initiating events, and in many cases adverse outcome, based on chemical features of a molecule. This practice is most well developed for agents that are covalently reactive (*Ellison et al., 2009; Yang et al., 2008*), but agents that act via weak interaction (e.g., receptor binding, reversible enzyme inhibition) also have characteristic chemical features. It is becoming possible to organise these into an ontology of molecular initiating events or modes-of-action, organised by chemical feature and by biological activity. An ontology of this sort would be the foundation for a more generalised and systematic approach to read-across, as well as for adverse outcome pathway development.

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3 THE SEURAT-1 RESEARCH STRATEGY: PROVING CONCEPTS

Maurice Whelan, Michael Schwarz, and the Scientific Expert Panel of the **SEURAT-1** Research Initiative.

'No experiment can prove me right. Only one proves me wrong'

Albert Einstein

3.1 Introduction

The first year of **SEURAT-1** saw the formulation of the SEURAT vision:

'*... to fundamentally change the way we assess the safety of chemicals by superseding traditional animal experiments with a predictive toxicology that is based on a comprehensive understanding of how chemicals can cause adverse effects in humans. To achieve this vision, the overarching SEURAT research strategy was also defined - to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health and to use this knowledge to develop complimentary theoretical, computational and experimental (in vitro) models that predict quantitative points of departure needed for safety assessment'* (*Whelan & Schwarz, 2011*).

This was followed by an elaboration of the strategy to aid in the translation of mode-of-action concepts into research practice (*Whelan & Schwarz, 2012*) and the definition of cluster-level objectives:

- formulate and implement a mode-of-action based research strategy for repeated dose systemic toxicity;
- develop new predictive toxicology tools and methods that are relevant for regulatory decision making;
- **demonstrate proof-of-concept at multiple levels - knowledge, systems, application;**
- provide the blueprint for applying the strategy to other chemical, toxicological and regulatory domains.

The objective to demonstrate proof-of-concept was put forward at the very first meeting of the **SEURAT-1** Scientific Expert Panel (SEP) held in February 2011 and is considered a central pillar of the strategy. The SEP believes that taking a proof-of-concept approach (i) aids in the identification of gaps in knowledge and research; (ii) facilitates effective communication and dissemination of **SEURAT-1** successes; and (iii) illustrates clearly how **SEURAT-1** tools and methodology can be applied in practice. Moreover, the SEP is of the view that although the projects are producing extremely valuable deliverables within their individual work programmes, cluster-level cooperation towards proof-of-concept will highlight the added value of having a consortium of complementary projects working together, and will show that ultimately the 'whole is greater than the sum of the parts'.

When elaborating this proof-of-concept objective, it was appreciated that key **SEURAT-1** concepts could be understood on different scientific levels, as summarised in *Figure 3.1*. On the first level, or the 'Knowledge' level, the proof-of-concept involves showing how a

systematic and practical process can be defined and employed to harvest mode-of-action knowledge and to organise and present it in a structured and efficient manner so that others can readily apply it. Since a wealth of information already exists on mechanistic toxicology, much of this exercise is focused on harnessing the available knowledge via systematic review of relevant literature, complemented with prospective computational and *in vitro* studies where needed. On the second level, or the 'Systems' level, the emphasis is on demonstrating how to assemble, calibrate and evaluate a system for predicting various aspects of chemical toxicity. Central to this exercise is the use of mode-of-action knowledge to rationally design these systems so that each component is relevant and contributes to achieving the prediction goal. On the third and final level, or the 'Application' level, the focus is on decision-making and how information derived from prediction systems that identify and characterise toxicological hazards can be used to the greatest effect when evaluating the safety of a chemical.

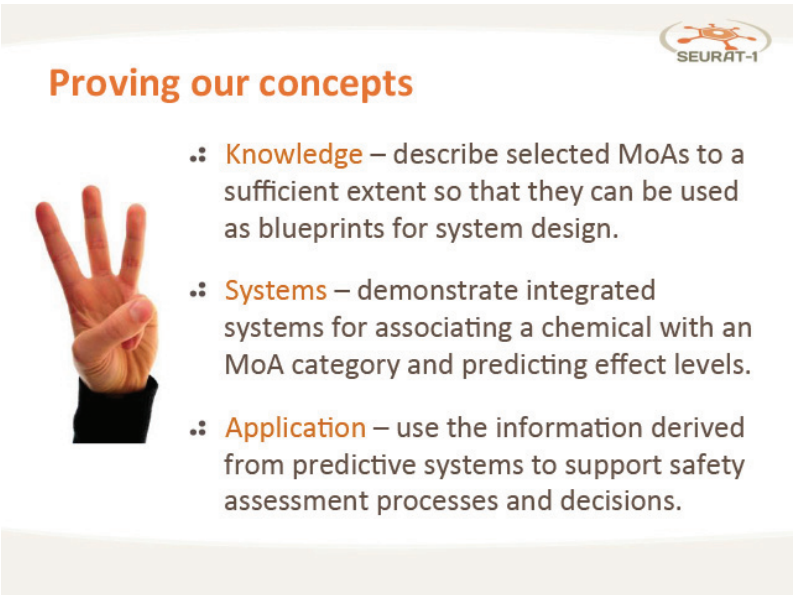

Figure 3.1 SEURAT-1 *is undertaking proof-of-concept at three different levels.*

In a large consortium such as **SEURAT-1**, there are a number of ways to address a proof-of-concept and so it was decided to define different case studies for each level. The nature of each case study dictates the level of cross-cluster engagement required, with some case studies relying heavily on multiple inputs from many partners, projects and working groups, while others are sufficiently served by smaller teams. Moreover, since the three proof-of-concept levels are interrelated, studies defined at different levels are likely to be complementary and thus benefit from mutual interaction.

3.2 Knowledge Level: Describing Mode-of-Action

As described previously (*Whelan & Schwarz, 2011*), the mode-of-action (MoA) framework (*Boobis et al., 2008*) is based on the premise that an adverse health effect caused by exposure to an exogenous substance can be described by a series of causally linked key events manifesting at different levels of biological organisation. When attention is restricted primarily to early toxicological events at the molecular and cellular levels, 'toxicity pathway' (*NRC, 2007*) is the preferred term for the series of key events. Although conceptually similar to MoA, an Adverse Outcome Pathway (AOP) is typically more expansive in nature (*Ankley et al., 2010*) and is triggered when the properties of an endogenous chemical and its bioavailability are sufficient to activate the related 'molecular initiating event' (*Figure 3.2*). Essentially, an AOP is reductionist at the process level, describing a particular scenario of how a biological system can fail due to the action of an endogenous chemical. Thus, an AOP does not describe the normal functioning of a biological system *per se*, but instead the sequence of events leading to its deterioration and dysfunction. However, a prerequisite for developing an AOP is sufficient understanding of the underlying normal physiology and the conditions that govern homeostasis. Such understanding is critical, for example, to put a key event such as cell apoptosis in perspective, that is, when can it be considered as a natural and necessary event in a healthy organism as opposed to a deleterious event on the road to an adverse health outcome.

Figure 3.2 *A simple linear depiction of an adverse outcome pathway, where the bioavailability of an endogenous chemical, at sufficient concentration and duration, triggers a chain of causally linked (key) events that ultimately leads to adversity.*

From the outset of **SEURAT-1**, aspects of identifying and describing MoA in the context of the **SEURAT-1** Research Initiative have been carefully considered (*Whelan & Schwarz, 2011*), aided through a dedicated workshop ('Mechanisms underlying repeated dose systemic toxicity', Ispra, November 2011; report available in *Gocht & Schwarz, 2012*), and the formal establishment of a Mode-of-Action Working Group (MoA WG) at the second **SEURAT-1** Annual Meeting (February 2012). Following a proposal from the SEP, the MoA WG decided to use the

OECD Guidance and Template for 'developing and assessing the completeness of adverse outcome pathway' (*OECD, 2012*) as a basis for capturing knowledge on MoA associated with repeated dose systemic toxicity. Specific organ toxicity (i.e. liver, heart and nervous system) was prioritised to best serve the immediate needs of the cluster.

The OECD AOP template clearly indicates which information should be provided, both to describe the toxicological process itself and the evidence that supports the postulation of the associated Key Events (KE), including the Molecular Initiating Event (MIE). What is lacking however is a practical guide on how to go about the actual task of developing an AOP. The MoA WG therefore adopted a learning-by-doing approach, which resulted in the emergence of a generic AOP development process, as outlined in *Figure 3.3*. The first attempt within **SEURAT-1** to systematically apply this approach involved the elucidation of two AOPs in the liver, namely, receptor-mediated steatosis and protein alkylation leading to fibrosis (*Landesmann et al., 2012*). The lessons learned during this exercise were shared with other AOP developers during the **SEURAT-1** workshop dedicated to 'Describing mode-of-action in liver toxicity using adverse outcome pathways' (Ispra, October 2012 – see chapter 4.10.5.3). There was consensus that although AOP development should be agnostic to begin with, at some point the intended use of the AOP needs to be taken into consideration to ensure that the ultimate product is fit-for-purpose. For example, an AOP intended to convey mechanistic understanding between scientific peers will differ from one intended to guide hazard identification, which in turn will differ from an AOP that should provide the basis for quantitative risk or safety assessment. In some respects therefore an AOP is never 'finished' but is developed to a point where it serves an intended purpose, with the purpose dictating how much detail and certainty is required from the developer. One aspect, which continually arises in this respect, is the challenge to include quantitative rather than purely qualitative information in an AOP in order to satisfy more demanding use-cases such as the development of integrated assessment and testing strategies for the prediction of *in vivo* points-of-departure.

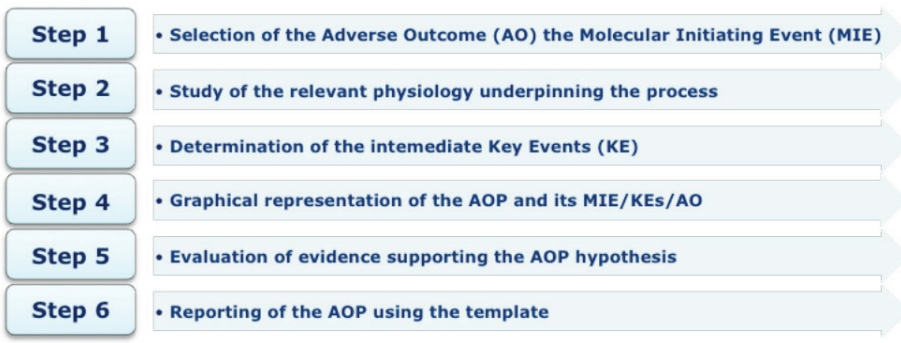

Figure 3.3 *A generic stepwise process to develop an AOP in line with the OECD template and guidance from the (OECD 2012).*

The development process outlined in *Figure 3.3* relies heavily on a systematic review of the literature to mine out the mechanistic knowledge applicable to the AOP in question. Considering the wealth of information already available, an AOP can be typically brought to a relatively mature state of development by studying relevant review papers and reported studies. However, at some point the process plateaus since the finer mechanistic detail specific to the AOP is often lacking in the description of third-party investigations. This is due to the fact that these investigations were usually conducted with different research objectives in mind. Thus, more extensive development of an AOP can require the undertaking of prospective experimental (*in vitro*) investigations that are specifically designed to shed light on the less understood aspects of the AOP. Such 'knowledge discovery' research is necessary, for example, to develop more precise descriptions of the dynamic relationships between KEs and to be explicit in quantitative terms about the conditions that dictate the transitioning from one event to another in the pathway.

The linear depiction of an AOP shown in *Figure 3.2* is useful to convey the basic concept of certain exposure conditions triggering a causally linked chain of events that ultimately leads to an adverse outcome. It is, of course, very simplistic in its representation of AOP dynamics. An expanded depiction of an AOP is shown in *Figure 3.4* which again is rather simplistic, but which endeavours to illustrate that an AOP ultimately unfolds or evolves at all levels of biological organisation in a parallel and continuous manner. In addition, this depiction conveys the fact that all the biological levels are dynamically interdependent, and thus an event on one particular level can trigger a consequent event at another level, either higher or lower.

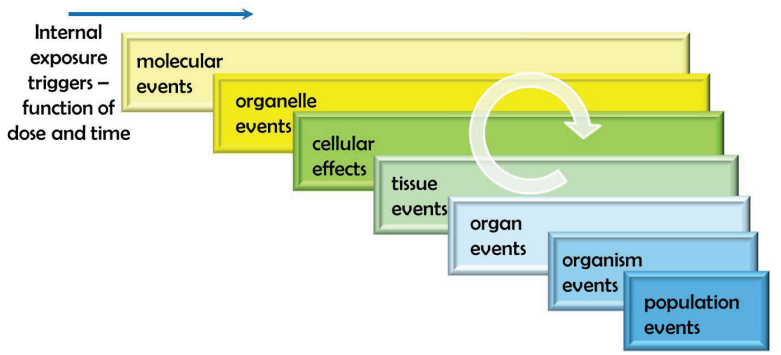

Figure 3.4 *An expanded depiction of an adverse outcome pathway that illustrates the parallel and continuous evolution of the AOP at different but interdependent levels of biological organisation.*

The **SEURAT-1** Research Initiative is adopting the AOP approach but is taking into consideration that the linear thinking, in terms of a 'causally linked chain of events', is over-

simplistic as biological, living systems (independent of whether they are organisms or cells in culture) are reacting as a 'network of events' and, therefore, may be better represented by thinking in terms of systems ('systems toxicology'). In principle, this is well described by understanding toxicological effects as a perturbation of homeostasis, or the establishment of a new homeostasis following exposure to chemicals (*Hartung et al., 2013*). The challenge is then to identify the appropriate representative readout for such a perturbation in the reaction pattern ('key event'). However, in repeated dose toxicity, the evolution of the AOP is dependent on exposure to an endogenous agent over time which applies a continuous triggering or perturbation at the molecular level. As an AOP progresses, the effects caused by downstream events become manifest at progressively higher levels, eventually evident in the dysfunction of the whole organism and potentially a (sub-)population.

There is growing appreciation within the **SEURAT-1** community that the conception, design and execution of experimental investigations intended to explore toxicological mechanisms in support of AOP development is a challenging and resource-intensive undertaking that requires considerable expertise. Moreover, the experimental approach employed for AOP development is typically very different to that required for implementing a testing strategy that applies an AOP. It is clearly the case that discovering and charactering KE relationships and associated biomarkers of effect may require highly sophisticated experimental systems and data analysis, whereas once described, the test system that is built to screen a chemical against a KE using the identified biomarkers will be far simpler. Of course, the development and application of an AOP is ultimately an iterative process starting with an unbiased description of the knowledge available, a tailoring with respect to the intended use, followed by application and evaluation, and then further development and refinement.

Although experimental investigation to elucidate mechanisms is an important activity within **SEURAT-1**, there is widespread acknowledgement that mining publicly available databases reporting both *in vitro* and *in vivo* toxicity studies can provide an invaluable source of MoA information to support AOP development. In particular, there is an enormous amount of toxicogenomics data that, with the appropriate analysis, could uncover hidden mechanisms and events and provide supporting evidence for an AOP during its development and evaluation. This motivation led to the organisation of a **SEURAT-1** workshop in November 2012 in Tuebingen, entitled, 'Exploring existing data bases for modes-of-action of repeated dose systemic toxicity' (a detailed workshop report is given in chapter 4.10.5.4). The aim was to explore in practice how to mine the extensive information hidden in publicly available databases on acute and sub-chronic toxicity studies for defining relevant toxicity pathways. Databases that were given particular consideration were DrugMatrix® (US NIEHS National Toxicology Program) and TG-GATEs (Japanese Toxicogenomics Project). These databases contain information on organ toxicities of a large number of test chemicals as a function of exposure time and dose, but also information on global gene expression changes *in vivo* (typically rodent) and *in vitro* (both rodent and human cells).

One clear take-home message from the workshop was that query-formulation is critical to ensure an efficient and effective data-mining process. One needs to be clear upfront about the specific purpose of the data-mining exercise since this has considerable influence on the search strategies adopted and the manner in which the data is analysed and interpreted. Mining scenarios can include, for example, the discovery of mechanisms or modes-of-action of a chemical with unknown toxicological properties, the confirmation of events or inter-event relationships associated with an AOP, the description of the time-course of AOP events, the grouping of chemicals which demonstrate similar molecular pathway signatures, or the comparison of pathway information for a particular chemical between *in vivo* and *in vitro* studies, and between species. Another important conclusion was that although gene-expression pattern analysis is indeed an informative step in the process, functional interpretation of expression patterns through the study of gene annotations and the association of genes with pathways was an essential step towards mechanistic understanding. In addition, due to the nature of typical transcriptomics studies, the conclusions drawn from any data-mining exercise will likely require some level of confirmation with complementary assays and biomarker analysis (e.g. at the level of protein or cell function).

The MoA WG has recently developed an AOP for cholestasis resulting from the inhibition of the bile salt export pump, to complement the AOPs for steatosis and fibrosis. There are, of course, numerous other organ-specific adverse effects associated with repeated dose systemic toxicity and so to go beyond the liver, the MoA WG organised a workshop in March 2013 in Ispra entitled, 'Using mechanistic information in developing the concept of the adverse outcome pathways relevant to human neurotoxicity evaluation' (see chapter 4.10.5.6). The primary aim of the workshop was to map out well established pathways of neurotoxicity that are supported by strong scientific evidence, and in doing so, to discriminate between human neuropathologies and pathways that are relevant to adulthood and those associated with more vulnerable life stages, such as development and ageing. An additional aim of the workshop was to propose cell models, biomarkers and reference chemicals that could be used to design *in vitro* assays linked to key events within pathways. The intention is to publish the findings of the workshop in a report that will include a brief outline of the AOPs identified and considered for further development.

The OECD guidance and template for AOP development and evaluation were used to structure the workshop discussions and output. Key events considered included NMDA-R over-activation, NMDA-R inhibition, mitochondria dysfunction, inhibition of NTE, interference with GABA-R and sodium channels, and neuro-inflammation. As in the previous MoA related workshops, it was appreciated that AOP development is very much context-dependant. Factors such as life-stage, sex, time or window and duration of exposure, biokinetics and species specificity all need to be explicitly considered and discussed during the AOP development and evaluation process. After an initial mapping of the AOP landscape, it became apparent that although there are indeed a plethora of low-level mechanisms related to neurotoxicity,

there was evidence to indicate that one could possibly screen for broad neurotoxicity hazard considering a rather modest number of key events. Regarding the design of *in vitro* assays that address these key events, it was felt that the test system or biological model should comprise different neuronal cell types mixed with glial cells (i.e. astrocytes, microglia and oligodendrocytes) since they play a critical role in an organism's response to chemically induced neurotoxicity.

A lot of experience has been gained within the **SEURAT-1** Research Initiative on AOP development and the effort is bearing fruit. A generic process for AOP development is in place that provides a practical way to proceed that is in line with the OECD AOP reporting template and guidance. However, further development of the AOP framework is needed, for example on aspects such as standardisation of toxicological terms based on selected ontologies, and more effective ways of graphically illustrating AOPs including accurate and understandable representations of the dynamic relationships between key events. The development of web-accessible knowledge bases for facilitating a community approach to AOP development and evaluation, to crowd-source and 'compute' knowledge, would be vastly superior to the current approach of simply exchanging documents and would improve the efficiency of the whole process enormously. There are initiatives on-going at the OECD to address these issues and thus it is possible that **SEURAT-1** could benefit from any progress made in the short and medium term.

3.3 Systems Level: Toxicity Prediction

Demonstrating proof-of-concept at the knowledge level is all about systems diagnostics and reverse engineering in order to describe a compendium of AOPs that cover the toxicological landscape. AOP development can be seen too as an 'inverse problem', where system dysfunction is studied in order to understand normal system dynamics. Toxicity prediction on the other hand is a direct problem, where AOP knowledge is exploited for the rational design of integrated systems intended for specific prediction goals.

This proof-of-concept exercise was launched at the 3rd **SEURAT-1** Annual Meeting (March 2013) with a first step of achieving a common understanding of the task at hand. The aim was to align our conceptual thinking in relation to toxicity prediction and to start teasing out the type of case studies that might be considered. In addition, the annual meeting afforded the opportunity to agree on the process and practicalities of elaborating and executing case studies.

The overall design philosophy proposed for this proof-of-concept is shown in *Figure 3.5*. It suggests that the most effective systems will be those that are designed to address specific prediction goals, and for which the relevant mechanistic knowledge (captured within an AOP for example) has been used as a blueprint for the optimal combination of complementary methods. In reality, getting the right balance between these three essential elements to 'hit the sweet spot' can be considered as a problem of 'constrained optimisation' since any gaps in MoA knowledge or lack of suitable methods will restrict the scope of prediction.

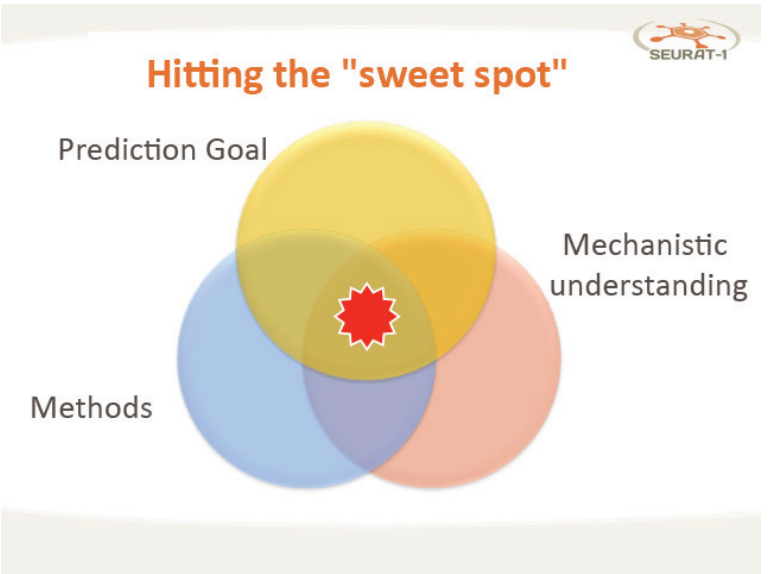

Figure 3.5 *Illustration of the design philosophy proposed for the development of integrated systems for predicting toxicity, where optimal solutions ('sweet spot') depend on using mechanistic understanding of the toxicity of interest to select and combine the right methods to address a specific prediction goal.*

One must design for purpose to be ultimately successful, but being explicit about a prediction goal is not as straightforward as it sounds. There are, in fact, many types of prediction possible, in theory at least. These could include:

- profiling the bioactivity of a chemical to associate it with an activity category that is related to a toxicity endpoint;
- identifying a chemical as being associated, or not, with a MoA that is likely to cause an adverse health outcome;
- ranking chemicals within a bioactivity or MoA category with respect to their potency or likelihood to cause toxicity;

➠ predicting a quantitative *in vivo* point-of-departure for a chemical with respect to a specific toxicity endpoint.

Mechanistic understanding comes from knowledge of mode-of-action. One can make useful predictions without necessarily having deep mechanistic understanding, but the level of understanding will influence both the predictivity of a system and the associated uncertainty. Moreover, end-users and decision makers often have difficulty accepting systems that lack a clear mechanistic basis even if there is empirical evidence of good predictive performance.

There is no shortage of innovative methods in **SEURAT-1** since many project deliverables are geared towards *in vitro* or computational method development, as reflected in one of the cluster-level objectives. Towards the end of 2012, a survey was conducted across the consortium which identified and described over 40 methods that are under development. These will be included in the **SEURAT-1** *Method Catalogue*. During this survey, an attempt was made to annotate each method with respect to the mode-of-action it may relate to, to make it more readily available for inclusion within an integrated prediction system. **SEURAT-1** methods include, for example, a range of pathway-based *in vitro* assays built around human cells and pathway-specific biomarkers, bioreactors that reflect tissue-level events, biokinetics models for *in vivo* to *in vitro* extrapolation, and computational models of receptor-binding and molecular networks.

To better understand this design philosophy, one can explore the three overlapping regions between two of the three fundamental elements (*Figure 3.5*). First, with a toolbox of methods and a prediction goal in mind, but without any mechanistic understanding, system development will likely involve a trial-and-error process that relies on statistical modelling to indicate the best method combinations. If a system is found to perform well, then post-development analysis of its components can of course aid in gaining mechanistic understanding. This scenario reflects a traditional paradigm that has worked to some extent with the less complex health effects, such as those associated with topical toxicity, but which can struggle when addressing complex systemic effects. Second, having a clear understanding of the toxicity that one wishes to predict and a well-defined prediction goal, but no suitable methods, then system development remains on the drawing board. This can be the case, for example, when the toxicity in question involves complex interactions between multiple cell types that cannot be reproduced with simple biological models *in vitro*. In this scenario, however, the specifications for the method or test system required should be clear and can be used to guide development. Ideally, a rational design process would be followed in this case, as previously described (*Whelan & Schwarz, 2012*). Third, having a suite of methods and ample toxicological knowledge, but no clear prediction goal usually results in underutilisation of technical and intellectual resources for the purposes of safety assessment. Such a scenario is not uncommon in basic research, where often the focus is on the development and use of methods to discover mechanistic knowledge, rather than to apply the available knowledge and tools to make predictions.

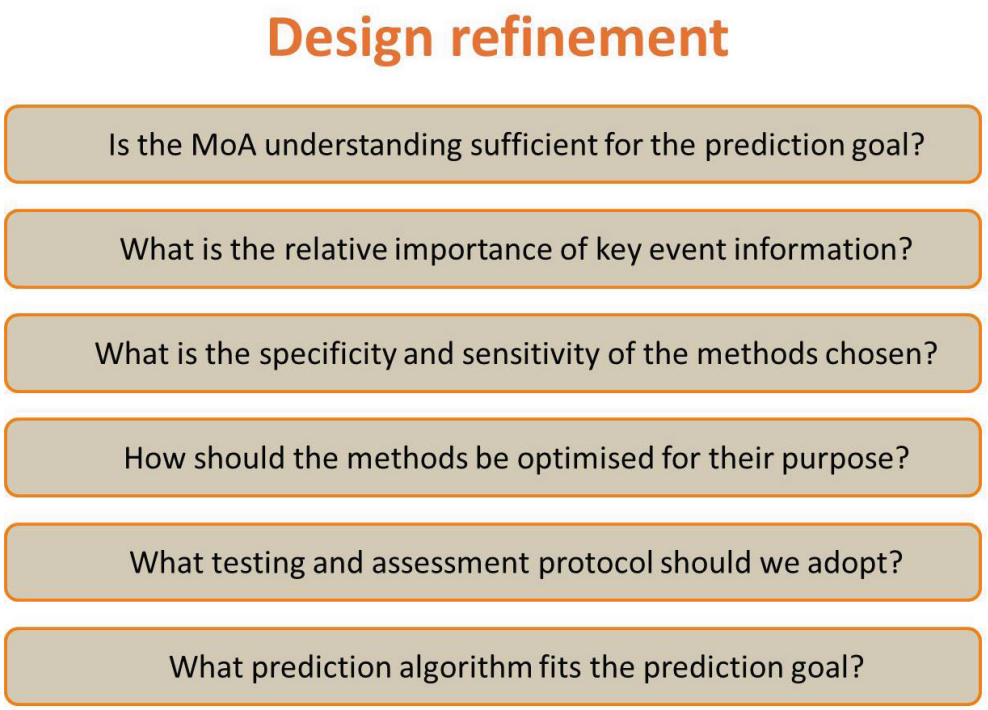

Figure 3.6 *Some of the important factors to be considered when refining the design of an integrated system to improve its prediction performance.*

The design and development of an integrated system for predicting toxicity is an iterative process. Design refinement towards better predictive precision and accuracy comes from consideration of many factors, including those listed in *Figure 3.6*. The initial step, however, is probably the most difficult i.e. scoping out the toxicity prediction case study for the first time and trying to match up the prediction goal with existing mechanistic knowledge and suitable methods. In order to provide an efficient and harmonised basis for analysis and discussion of candidate case studies between partners and within the SEP, it was decided to use a template to capture essential information in a structured manner. An extract of this case study description template is shown in *Table 3.1* which asks the proposer to define the prediction goal, to describe the mechanistic basis of the system, the scientific approach being employed for the design and development, and the reference chemicals that will be used for system development, calibration and evaluation.

Table 3.1 *An extract of the template that is being used to propose case studies that address the proof-of-concept related to predictive toxicity.*

Prediction Goal	Please clearly indicate the purpose of the system/strategy/ method being developed with respect to the toxicological effect(s) it is designed to predict.
Mechanistic basis	Please describe the mechanistic basis underpinning the approach, mentioning for example any relevant mode(s) of action that are being considered.
Scientific approach	Please briefly describe the scientific approach to be taken including for example the design rationale, what methods will be employed, the basis for predicting an outcome and interpretation of data, and if foreseen, any anticipated validation activity to assess predictive performance.
Chemical selection	Please identify what chemicals you intend to use for development and assessment of the system, including for example positive and negative reference chemicals.

The definition of predictive toxicity case studies is by no means a trivial task. It takes time and a considerable amount of discussion to strike the right balance between ambition and feasibility. Formulating and presenting a predictive toxicity case study as a 'problem to be solved' will help grab the attention of potential contributors and motivate them to get involved. Execution of a case study has significant resource implications and thus will essentially depend on being able to exploit activities and deliverables already foreseen in the project work programmes. Inevitably, case study proposals are likely to be lacking in certain elements, but the attitude being adopted is that 'gaps are there to be filled'. The SEP has an important role to play in this respect and can look across the entire cluster to identify how any deficiencies in resources, expertise or methodology regarding a promising case study can be addressed.

3.4 Application Level: Safety Assessment

This proof-of-concept is being led by the Safety Assessment Working Group of the **SEURAT-1** Research Initiative (see chapter 4.10.8). It is closely related to the primary aim of this Working Group – to develop an 'Integrated Assessment Framework' (IAF) that can be used as a basis for the rational combination of information derived from predictive tools to support a safety assessment process or decision to achieve a stated protection goal. The ambition is

to propose an IAF during the course of **SEURAT-1** that can undergo some initial practical evaluation in the context of assessment strategies specific to repeated dose systemic toxicity. It is hoped and anticipated, however, that the IAF will be further developed and refined beyond **SEURAT-1**, eventually being taken up by industry to, for example, assess a new ingredient in a cosmetic product, set priorities for evaluation, or to substitute for a repeat-dose test in rodents to meet particular regulatory requirements.

The proof-of-concept was specifically addressed by the Safety Assessment Working Group during its workshop held in Tuebingen in November 2012 entitled, 'The development of case studies to define fit for purpose safety risk assessment of repeated dose systemic toxicity' (see chapter 4.10.8.2). As a result, two different case studies were proposed that reflect two typical safety assessment scenarios that the IAF will need to facilitate. The first case study is probably the most ambitious – Quantitative Safety Assessment (QSA). The objective is to arrive at a point-of-departure or reference value for a particular chemical, that can be used as a basis for a safety decision, by conducting an *ab initio* assessment using only the methods available within the **SEURAT-1** Research Initiative. This QSA case study will bring many aspects of **SEURAT-1** into play and will explore how far one can actually go with the tools and methodology that are available now. Of course, the exercise is also designed to highlight major gaps and shortfalls that hinder a comprehensive and credible QSA, thereby providing a clear indication on where future research and development efforts in the field of safety assessment need to focus.

The second case study deals with Chemical Categories (CC) and Read-Across (RA) which are approaches probably best known for hazard and safety assessment of industrial chemicals under REACH, but which are often employed in other sectors too. The purpose of this case study is to demonstrate how information on a chemical generated using **SEURAT-1** methods can be used to associate it with a chemical category and to deduce or predict its hazard properties by 'reading across' from the properties of other chemicals belonging to the same category. The intention is to use both toxicokinetic and toxicodynamic data derived from computational and *in vitro* studies to establish and support a CC and RA argument. Of particular interest in this case study is to show how traditional approaches based primarily on consideration of chemical structure can be supplemented with mechanistic information derived from computational methods and *in vitro* '-omics' experiments to either support or reject a CC or RA proposal. The Safety Assessment Working Group considers this a realistic target for **SEURAT-1** and expects that the results will demonstrate how the output of the cluster can find immediate use for supporting chemical safety assessment with the potential of reducing animal testing.

3.5 Conclusions and Outlook

Considerable progress has been made on all levels. There is widespread engagement across the cluster and mutual understanding of the importance of achieving the proof-of-concept objective. There is already evidence of the impact of some of the earlier MoA case studies i.e. some projects are tailoring their activities to benefit from the mechanistic knowledge that was gathered and communicated.

The definition and execution of the case studies on all three levels is very inclusive in that the partners, projects, working groups, the SEP, and industry advisors are all involved and contributing to the process. Thus the proof-of-concept exercise is driving further integration and cooperation within the cluster and creating a sense of unity in showing the world what the **SEUART-1** Research Initiative can do. The proof-of-concept case studies will also provide a strong basis for practical engagement with external cooperation partners such as the OECD Advisory Group of Molecular Screening and Toxicogenomics, the Mode-of-Action Steering Group of the WHO International Programme on Chemical Safety, the European Chemicals Agency, the US EPA ToxCast programme, the Cosmetics Europe task force on systemic toxicity, the European Platform for Alternative Approaches to Animal Testing, and the Tox21 consortium.

The focus in the short term will be on further elaboration and discussion of cases studies based on feedback provided by contributing partners and the SEP. This will lead to the formulation of case study implementation plans with the aim of completing the main body of work before the end of 2014. The next annual meeting in early 2014 will therefore coincide with the midway point of the proof-of-concept exercise and thus the SEP has already anticipated that this meeting will focus heavily on reviewing the progress made. Although it is still relatively early days, the outlook is an optimistic one since when endeavouring to prove concepts, commitment to the process itself is as important as the eventual result.

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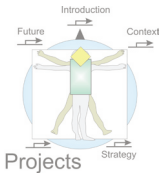


4 THE PROJECTS

"Start by doing what's necessary, then what's possible, and suddenly you are doing the impossible"

St. Francis of Assisi

4.1 Introduction

This chapter provides a comprehensive overview of the projects of the **SEURAT-1** Research Initiative and, thus, generates the backbone of the Annual Report. Overall, the **SEURAT-1** Research Initiative is designed as a coordinated cluster of five research projects supported by a 'data handling and servicing project' and a 'coordination and support project' at the cluster level.

The following integrated projects form the core of **SEURAT-1**:

- 'Stem Cells for Relevant efficient extended and normalized TOXicology' (*SCR&Tox*): Stem cell differentiation for providing human-based organ-specific target cells to assay toxicity pathways *in vitro;*
- 'Hepatic Microfluidic Bioreactor' (*HeMiBio*): Development of a hepatic microfluidic bioreactor mimicking the complex structure and function of the human liver;
- 'Detection of endpoints and biomarkers for repeated dose toxicity using *in vitro* systems' (DETECTIVE): Identification and investigation of human biomarkers in cellular models for repeated dose *in vitro* testing;
- 'Integrated *In Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety' (COSMOS): Delivery of an integrated suite of computational tools to predict the effects of long-term exposure to chemicals in humans, based on *in silico* calculations;
- 'Predicting long-term toxic effects using computer models based on systems characterization of organotypic cultures' (NOTOX): Development of systems biology tools for organotypic human cell cultures suitable for long-term toxicity testing, and the identification and analysis of pathways of toxicological relevance;
- 'Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology' (ToxBank): Data management, cell and tissue banking, selection of 'reference compounds' and chemical repository.

Furthermore, a coordination action project was designed in order to facilitate cluster interaction and activities:

- 'Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals' (COACH): Cluster level coordination and support action.

All of the projects started on 1 January 2011. The first volume of the Annual Report focused on the plans and challenges of the different projects and the second volume contained first results from the research conducted within the **SEURAT-1** Research Initiative. This is continued in

this third volume, which presents the research highlights from the second year in the context of the overall progress within the projects. Furthermore, each project description includes the following sections: (i) the innovative aspects with respect to the achieved results; (ii) the established cooperation with other projects in the **SEURAT-1** Research Initiative; (iii) the expected progress within the third year of the project; and (iv) future perspectives in the long run, describing possible next steps based on achieved and expected results from the various projects. An overview of the Principal Investigators from each institution, organised within the projects, completes these sections.

A section summarising meeting reports at the project- and cluster-levels follows the detailed project descriptions. It also contains extended abstracts from the awardees of a poster session organised at the third Annual Meeting of the **SEURAT-1** Research Initiative. Overall, this section provides a transition from the level of the various projects to the cluster level and, consequently, is followed by a report on cross-cluster cooperation.

These cross-cluster activities are emerging more and more over the lifespan of the **SEURAT-1** Research Initiative, which is reflected by the increasing volume of this section. The second volume of this Annual Report described the *modus operandi* of cross-cluster Working Groups as the central elements for facilitating cooperation between projects and people. A central new aspect is now the development of a **SEURAT-1** roadmap as a tool to monitor progress towards the achievement of the cluster-level objectives. This is extensively reported in this third volume. Of course, the Working Groups play a major role in these efforts and reports on activities and workshops conducted under the umbrella of the Working Groups complement this section. In total, the following six Working Groups are active: (i) the Gold Compounds Working Group, (ii) the Data Analysis Working Group (these two have been active since the beginning of the **SEURAT-1** Research Initiative), (iii) the Mode-of-Action Working Group, (iv) the Biokinetics Working Group, (v) the Stem Cells Working Group and (vi) the Safety Assessment Working Group (the latter four were established during the second Annual Meeting and started their activities last year). All Working Groups are populated with members from different projects, enabling targeted discussions on the needs and contributions of the **SEURAT-1** research projects to meet the cluster-level objectives. Additional workshops were organised based on identified needs from the projects, which are not all addressed by the Working Groups, and respective reports about these activities are also included in this section.

Finally, a report describing the outreach activities finishes this chapter. The central aspects here are: the organisation of the **SEURAT-1** summer school (the first was held in 2012 and a summary report is given in this section); dissemination activities on conferences; a brief overview of awards and prizes won through **SEURAT-1** research activities; and the **SEURAT-1** public website. A highlight from last year's activities was the production of a video outlining the aims of the NOTOX project in the context of the **SEURAT-1** Research Initiative (the video is publicly available on the NOTOX project webpage: http://www.notox-sb.eu/film). Besides the Annual Report, the activities mentioned above are the most important cluster-level tools to promote the dissemination of knowledge.

4.2 SCR&Tox: Stem Cells for Relevant efficient extended and normalised TOXicology

Andrea A. Robitzki, Heinz-Georg Jahnke, Tamara Friedriszik, Stephan Fleischer, Diana Seidel, Vania Rosas, Marc Peschanski

4.2.1 Introduction and Objectives

The need for a profound shift in the way toxicology testing is carried out for chemicals in the pharmaceutical and cosmetic industry is clearly acknowledged by all, in both the industry and academia as well as in institutional bodies. Change is inevitable because the current system is not based on fundamentally sound science, but rather on descriptive data from high dose animal tests. The extrapolations – across species, from high test doses to low exposures, and from descriptive endpoints in animals to their possible human correlates – are handicapped by the lack of underlying mechanistic information. Although this has been often instrumental in the past, it has sometimes also shown to be clearly unreliable. In addition, our current approach is too expensive and too slow, capable of only limited throughput (*Kramer et al., 2007*).

A number of expert reports and publications now call for reorienting testing to the molecular level, highlighting the concept of 'toxicity pathways' within human cells that would be triggered by a toxicant exposure at a low dose that, by itself, does not provoke major cell toxicity but induces changes in cell homeostasis to cope with the phenomenon (*NRC, 2007; Hartung, 2009*). Repetition of exposure, or increase in dosage, may eventually lead to actual irreversible changes and severe consequences. Evaluation of toxicants calls, therefore, for new models to be created that will allow for assessing toxicity pathway responses *in vitro*, that will deliver a more accurate profile of acute toxicity in humans and, possibly, also reveal more subtle chronic toxic contraindications. Moreover, at a point in time when pharmacogenomics are becoming one of the major drivers toward personalised medicine, there is general agreement that predictive toxicology needs to take into consideration human gene polymorphisms (*Katz et al., 2008*). Implementation of this new strategy based upon *in vitro* tests requires the most relevant and reliable model systems, which should also be robust and scalable in order to be instrumental on an industrial scale.

Pluripotent stem cells, whether of embryonic origin (ES cells) (*Thomson et al., 1998*) or induced to pluripotency by genetic re-programming of somatic cells from donors (iPS cells) (*Takahashi et al., 2007; Takahashi & Yamanaka, 2006*), share a number of attributes that, in our view, make them uniquely suitable for meeting the challenges of the new toxicity testing paradigm. These cells – of human origin – are either physiological (ES) or else apparently similar to physiological cells (iPS), thus providing some guarantee for relevance (*Hoffman & Carpenter, 2005; Yu and Thomson, 2008*). Because they are formally immortal, they can be obtained in any requested amount from any chosen donor. Repeatability of testing on a single genetic background is thus perfectly feasible. They can also be obtained in similar phenotypic conditions from any number of different donors, opening the path for studies of a potential inter-individual variability of responses. Pluripotent stem cells are, by definition, amenable to differentiation into almost any cell type, of any lineage, at any stage of their maturation, whenever one has identified a workable protocol for *in vitro* processing of the cells. It is, in particular, possible to obtain not only fully differentiated cells of any organ but also intermediate precursors. Those precursors have often proved quite interesting for long-term scalable analyses because they can be maintained for many passages (e.g. over 100 for human ES-derived neural precursors) without loss of lineage-specific traits and may, therefore, be instrumental for analysis of repeated-dose toxicity. Pluripotent stem cells can be used for parallel analysis of the effects of toxicants on cells representing different organs of interest, on an identical genetic background. They are also discretely amenable to genetic engineering either at the undifferentiated stage or as self-amplifiable intermediate precursors, allowing for provision of specific properties of interest, such as gene constructs indicative of the action of chemicals or else transcription or signalling factors promoting desired phenotypic changes. The ***SCR&Tox*** programme is, therefore, entirely based upon human pluripotent stem cell lines. It will analyse in parallel human ES and iPS cells because of their complementary interest; the former being already much more studied and understood and having in particular demonstrated robustness and reliability on an industrial scale, the latter being potentially more versatile, in particular for large-scale analysis of the impact of human polymorphisms on responses to toxicants.

The aim of the ***SCR&Tox*** program is to provide the biological and technological resources needed to assay toxicity pathways *in vitro* and to demonstrate on industrial platforms that these resources can be reliably and robustly implemented at the required scale. The programme has been organised in two sequentially scheduled parts of equal duration, dedicated to the provision of biological and technological resources, and to demonstrating the value of the paradigm, respectively.

For the first half of the programme (first two and a half years), the scientific objectives of the proposal are:

- to obtain the pluripotent stem cell lines required, both in terms of quality, i.e., ES and iPS, from a sufficient number of donors, and quantity through

implementation of scalable production technologies;

➠ to design and implement optimal protocols for differentiation of pluripotent stem cells along five different lineages (liver, heart, CNS, epidermis and muscle), to terminally differentiated cells. Some of the cell types will be characterised in an additional, intermediate precursor stage;

➠ to design and implement engineering methods to optimise those differentiated cells specifically for toxicity pathways assays;

➠ to identify, optimise and standardise technologies for exploring cell functions relevant to toxicity pathways assays.

For the second half of the programme (second two and a half years):

➠ to implement on the bench cell-based assays of toxicity pathways using optimised and newly developed technologies;

➠ to promote biological resources for scale, reliability and robustness for implementation on industrial HTS platforms;

➠ to develop at least one stem cell-based assay of a toxicity pathway validated on the bench for implementation on industrial HTS platforms;

➠ to demonstrate the value of at least one prototype of a stem cell-based toxicity pathway assay on industrial HTS platforms;

➠ to enter at least one prototype of a stem cell-based assay of a toxicity pathway into normalisation and validation;

➠ to address the potential phenotypic diversity of cell lines and select a robust panel of cells for large-scale preparation of test cultures that are suitable for high-throughput screening.

4.2.2 Main Achievements and Challenges in the Second Year

The first part of the ***SCR&Tox*** project is dedicated to the provision of biological and technological resources required for assaying toxicity pathways, with direct impact on the second part in which a model system will make use of the biological and technological resources in order to demonstrate the value of the proposed paradigm for toxicology testing, up to an industrial scale.

Concerning biological resources, we have started to provide cell lines at the undifferentiated stage, with emphasis on a large diversity of donors in order to explore the impact of genetic polymorphisms on the responses to chemicals in humans. The iPS cell line reprogramming

is allowing us to select more readily donors with different ancestry backgrounds than ES cell lines. As an additional pre-requisite for development up to an industrial scale, we have established conditions for scaled-up production of large amounts of pluripotent cells. Because those cells can be amplified at will without ever entering into a senescence process, they are theoretically an unlimited resource for any requested amount of cells, a pre-requisite for industrial scale cell-based assays that will unavoidably require in excess of several hundred million cells per batch.

SCR&Tox is mastering this capacity by implementing the technologies required, using automated culture systems to grow cells in flasks and implement defined culture media for feeder cell replacement. Cells to be used for drug screening should reproduce phenotypic traits of relevant tissue cells. We have, therefore, sought to identify protocols for differentiating pluripotent cells into derivatives for each of five toxicological relevant lineages (liver, CNS, heart, skin and muscle). Protocols have been designed, except for muscle cells for which only an intermediate precursor stage is readily available but not a terminal differentiation. These protocols will now allow us to provide cells ready, quantitatively and qualitatively, for toxicology pathway assays in dedicated formats for direct use in the assay development and industrial-scale demonstration. This includes preparing 'ready-to-screen cell kits' for exploring all types of diversities: of chemicals, of dose dependency, of exposure times and repetition, of phenotypic sensitivity and of genotypic biases.

Concerning technological resources, we aim to provide all technologies and methodologies required for developing efficient cell-based assays seeking quantitative assessment of biomarkers associated to toxicity pathways. This involves validating the applicability on pluripotent stem cells derivatives of a multiparametric array of techniques analysing molecular components. We have first validated the implementation of classical '-omics' for gene and protein expression analysis in order to demonstrate the value of the biological resources. Large-scale evaluations of the status of gene expression and protein concentrations in cells allow understanding of the integrated biologic activities and can be used to catalogue changes after *in vitro* treatment with toxicants. We have started by testing on preliminary assays the usefulness of a differential transcriptomic analysis. The data retrieved from the RNA profiling have then been transferred for secondary analysis using interactomic methodologies. As a second step, we are developing a functional genomics screen approach that should allow us to identify dynamically genes that participate to pathways of toxicity. Large-scale screens for siRNA are on-going. In parallel, particular emphasis has been placed on bioelectronics (which is reported below as this year's selected highlight), leading to the standardisation of protocols that will be implemented in order to evaluate electrogenic changes associated with toxic exposure in cells that are physiologically electroactive, such as cardiomyocytes, muscle cells or neurons. These major achievements are based upon newly designed techniques allowing miniaturisation of such tests on cells in microarrays and microcavity arrays.

4.2.3 Selected Highlight: Bioelectronic Measurement Systems for Label-Free Monitoring of Cell Functionality

Introduction

Over the last decade, a vast amount of protocols were successfully developed for differentiation of hES cells to specific tissue cells. Moreover, within the last five years researchers followed the same approaches for using hiPS as the source of pluripotent stem cells. Especially for generation of cardiomyocytes and neurons there were tremendous improvements over the last years. In general, most differentiations lead to specific cell types that express typical early tissue-specific markers within days to weeks, raising a question about when a cardiomyocyte or neuron reaches the matured stage. More importantly, there is the question of at which stage of maturation it is possible to reliably predict a tissue-specific *in vivo* toxicity in an *in vitro* assay without high rates of false positive and/or false negative results.

In this context, today's stem-cell researchers are focusing on an accelerated maturation; for example, for cardiomyocytes and neurons. Furthermore, stability of stem cell-derived *in vitro* culture characteristics is of great importance, especially in the context of chronic/repeated dose toxicity testing. Although we are aiming for a paradigm shift towards marker identification for detection of chronic/repeated dose toxicity effects at an early stage, the validation of these novel *in vitro* toxicity assays can be achieved only by long-term analysis of the established cell culture models over days and weeks. When hES and/or hiPS cell-derived cells in particular are being used, a high degree of stability and standardisation must be guaranteed. Processes such as de- and trans-differentiation, or accelerated proliferation, have a high impact on genomics and proteomics and could critically alter marker expression or even identified toxicity pathways. Therefore, an intensive characterisation of stem cell-derived cell culture models is indispensable. In this context, label-free bioelectronic monitoring of differentiation processes and cellular functionality offer great opportunities for quality control and standardisation of specific cell type-based *in vitro* toxicity tests. In addition, the same techniques could be used for short- and long-term monitoring over days or weeks of cellular alterations induced by toxic compounds.

Impedance spectroscopy is widely-used to gain insight into cell properties and functions. Since it allows the non-invasive and label-free monitoring of cells and tissues several systems and approaches were developed for detecting cytotoxicity on cells. Moreover, the development of advanced impedance analysers allowed the fast and comprehensive analysis of cells, resulting in an electrical fingerprint of a well-characterised cellular model. Therefore, cellular processes, such as proliferation, apoptosis, cell motility, cell-cell and cell-substrate interactions, as well as activation of ion channels and signal cascades, can be sensitively detected. In a well-characterised electrode-cell/tissue system these different parameters can be distinguished and quantified. For electrogenic cells, a second technique offers the possibility to monitor the functionality and activity of cells. Using field potential recording

systems the action potentials of cardiomyocytes and neurons can be detected and traced over time. More strikingly, the recorded field potential gives detailed information about cellular electrophysiological characteristics. The detection of an action potential, the amplitude of the signal, the action potential duration and the shape of the electrogenic cell-derived field potential are suitable parameters that can be linked to distinct ion channel activity, or even to cellular subtypes, e.g., sinoidal, atrial or ventricular cardiomyocytes. The analysis of the electrophysiological parameters can be used to identify pathological cellular alterations, e.g., induced by toxicants. In the case of cardiomyocytes adverse side effects such as structural cardiotoxicity can be identified as well as arrhythmia-like QT syndrome-related prolonged repolarisation. Field potential analysis of neuronal network activity can give detailed insights into unwanted modulatory effects on neuronal activity. Since these bioelectronic techniques were established and used with matured cells such as primary cardiomyocytes and neurons obtained from animals, these multielectrode arrays have to be optimised and adapted to the extended demands of stem cell and stem cell-derived cell cultures.

Approach

In line with the ***SCR&Tox*** proposal, one focus is the development of bioelectronic monitoring systems for quality control of differentiation to specific matured target tissues and cells such as cardiomyocytes and neurons. Label-free bioelectronic monitoring systems are used for the sensitive detection of induced tissue-specific toxicity, especially with regard to the aimed chronic and repeated dose toxicity testing. Since stem cells and stem cell-derived cells have high demands on culturing conditions, the multielectrode array systems had to be tested and adapted according to these specific demands and the addressed differentiation processes. To achieve this goal, ***SCR&Tox*** partner University of Leipzig started with their developed multielectrode array and bioelectronic monitoring platform (*Figure 4.1*).

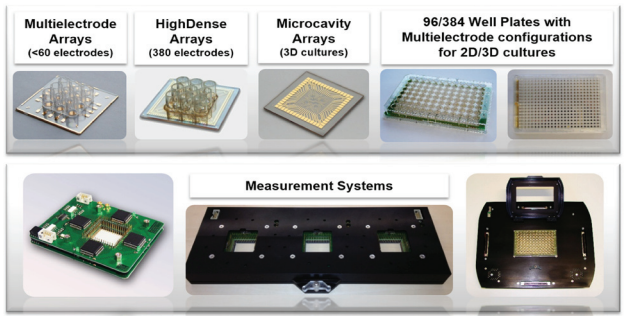


Figure 4.1 *Optimised multielectrode arrays for cell type-specific and assay-dependent sensitive monitoring of cell function and cellular alterations (top). For reliable and automated data acquisition, appropriate electronic boards were developed (bottom). Data source: University of Leipzig.*

The University of Leipzig group is the leading institution within ***SCR&Tox*** in the field of bioelectronic measurement systems. Using their expertise for optimising the cell-electrode interface for ultimate signal quality and detection sensitivity, they provided a broad range of multielectrode arrays for the different cell types and monitoring tasks. The group's array portfolio comprises large-scale electrode arrays for comprehensive monitoring of cell layers (e.g. stem cell colonies), microelectrode arrays for improved spatial resolution, as well as high-density microelectrode arrays with more than 380 microelectrodes per well for advanced monitoring of, for example, neuronal differentiation processes and neuronal network activity. More strikingly, the group at the University of Leipzig provided unique 3D-structured microcavity arrays that solve the problem of 3D culture monitoring. As the ***SCR&Tox*** project addresses the demand for an up-scaling of cell sources and assays, the University of Leipzig group provided their expertise in the field of large array production and integrated circuit packaging for up-scaling small-scale multielectrode arrays after optimisation and validation of the specific cell type and monitoring task to full format 96-/384-well plates. For reliable, automated monitoring of a wide range of multielectrode arrays and well plates, they provided suitable measurement systems with electronic boards optimised for minimum cross-talk and high signal-to-noise ratios as well as high precision impedance analysers and field potential amplifiers for the automated parallel recording of up to 24 multielectrode arrays. These platforms are mobile and, therefore, can be transferred to the partners within the ***SCR&Tox*** project that are working on the differentiation of specific cell types.

In order to get the most comprehensive information from one culture/sample, the University of Leipzig group wanted to combine several label-free bioelectronic monitoring techniques into one system. The parallel monitoring of electrophysiological activity by field potential recording as well as the dielectric properties of the cells and cultures by impedance spectroscopy offers a higher degree of cellular information and alterations, particularly in the case of electrogenic cells such as cardiomyocytes and neurons. Since these techniques have competitive demands on the properties of the electronic boards as well as the multielectrode arrays itself, state-of-the-art approaches generally offer separate monitoring platforms that prohibit a real parallel recording on the same electrode and, therefore, on the same cell (*Figure 4.2*).

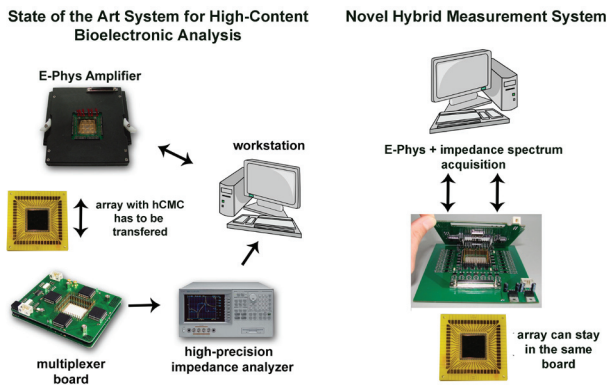


Figure 4.2 *Actual approaches for combined bioelectronic analysis use separate measurement platforms and require the transfer of multielectrode arrays and cultures between the measurement systems (left). With our novel hybrid measurement system we can record field potential as well as impedance spectra in parallel from the same electrode on the same array (right). Data source: University of Leipzig.*

Moreover, the arrays have to be transferred from one measurement system to the next and this increases methodic variations. To overcome this limitation, the University of Leipzig group provided their novel developed and patented hybrid measurement system, which integrates field potential recording and impedance spectroscopy monitoring within one electronic board (*Figure 4.3*). Using specific low-noise circuits in combination with a specially developed microcontroller for ultra-fast synchronised switching, the parallel recording of field potentials and impedance spectra from the same electrode is possible – providing a high signal-to-noise ratio as well as a high time resolution.

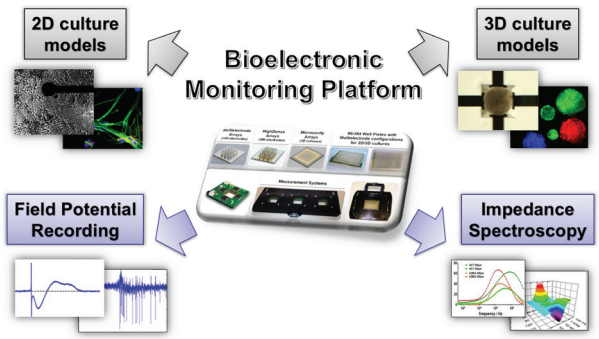


Figure 4.3 *Bioelectronic monitoring platform for the analysis of 2D- and 3D-culture models using field potential recording and impedance spectroscopy. This allows the functional monitoring of electrogenic cells (neurons and cardiomyocytes) including the detection of cellular effects due to toxic compounds. Data source: University of Leipzig.*

High-content bioelectronic monitoring system for hES-/hiPS-derived cardiomyocyte clusters: In close collaboration with ***SCR&Tox*** partner CSC/Cellartis, the University of Leipzig group developed a high-content label-free bioelectronic monitoring system for hES-/hiPS-derived cardiomyocyte clusters (hCMCs). The unique microcavity arrays that allow reliable analyses of 3D cultures were used for this purpose. The CSC/Cellartis group developed protocols for generating hCMCs that are highly enriched with cardiomyocytes and that offer superior electrophysiological characteristics and long-term stability. Since planar multielectrode arrays are not suitable for the sensitive monitoring of these clusters, especially in the context of long-term monitoring over days and weeks, the University of Leipzig group optimised their unique microcavity array technology to match the demands on the hCMCs and developed a novel microcavity array, which is based on laser microstructured fused silica substrates (*Figure 4.4*).

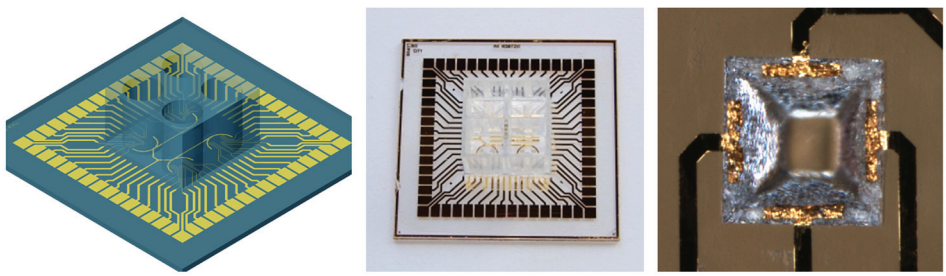


Figure 4.4 *Optimised microcavity array from the University of Leipzig group for monitoring of hES-/hiPS-derived cardiomyocyte clusters (in collaboration with CSC/Cellartis). This prototype is based on a microstructured transparent fused silica substrate with four wells and three microcavities per well (400 µm edge length). Data source: University of Leipzig.*

This microcavity array has optimised cavity sizes for the hCMCs in the range of 400–450 µm and is arranged in four independent wells and three cavities per well. This allows for the running of experiments with different treatments on the same array while obtaining information for each treatment in triplicate (three hCMCs per treatment). The whole design was done in accordance with the overall ***SCR&Tox*** up-scaling strategy; i.e., this well/array design can be extended to full 96-well plates.

Bioelectronic monitoring of stem cell-derived neuronal networks: A second focus was the development of a bioelectronic monitoring system for stem cell-derived neuronal networks, which was developed in collaboration with the ***SCR&Tox*** partners University of Bonn and Avantea. The University of Leipzig group monitored detailed functional and dielectric characteristics of neuronal maturation and alterations from stem cell-derived neuronal

networks using their high-density microelectrode arrays. Furthermore, a bioelectronic monitoring system for detecting long-term toxicity effects in a stem cell-derived muscle cell model is under development together with ***SCR&Tox*** partner I-Stem.

Results

High-content bioelectronic monitoring of stem cell-derived cardiomyocyte clusters: The great advantage of the microcavity array technology is that no cell adhesion on the electrodes is needed for obtaining a sufficient cell signal. Single hCMCs can be automatically placed in the cavities and instantly monitored (*Figure 4.5*, left). The microcavity surface for cell-repellent properties was modified in order to guarantee the integrity of the hCMC during long-term monitoring on the microcavity array. This offers a great flexibility for designing experiments with respect to studies addressing repeated dose toxicity, as the hCMC does not need to remain within the array in experiments running for extended periods of time (e.g., days or even weeks). In summary, the operator can chose the recording periods and durations according to the experimental protocol (e.g., repeated dosing).

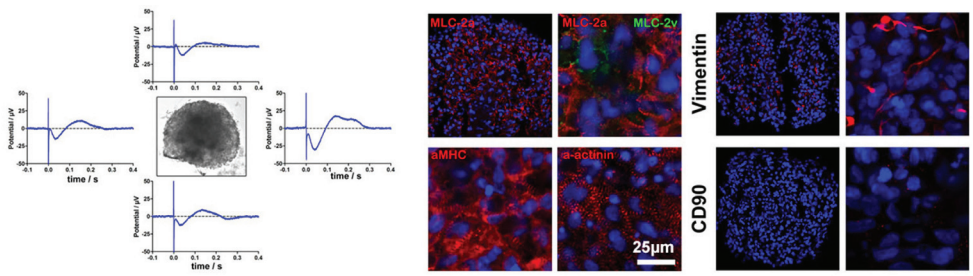


Figure 4.5 *The microcavity-based functional monitoring of hES-derived cardiomyocyte clusters allows the determination of beating frequency, excitation velocity and action potential duration (left). The validity of the obtained results depends on the quality of the cardiomyocyte clusters. The immunocytochemical characterisation of the developed hES-derived cardiomyocyte clusters revealed a highly enriched cardiomyocyte population with a high level of cardiac markers (MLC-2, aMHC, a-actinin), no fibroblasts (CD90) and only a small number of other mesodermal-derived cell types (vimentin) (right).*

A further advantage of the system is that the monitored hCMCs can subsequently be used for further analysis. The immunocytochemical staining after recording periods of 72 hours revealed hCMC cluster integrity comprising highly enriched cardiomyocytes (*Figure 4.5*, right). This was proven by the cardiac markers MLC-2a/v, aMHC and the correct contractile substructures (a-actinin). No fibroblast was found within the hCMCs (CD90) and only residual mesodermal-derived non-cardiomyocyte cells (vimentin) were observed.

Based on these promising results, we started long-term monitoring of hCMC functionality by field potential recording over 46 days (*Figure 4.6*). Using the developed hCMCs from the CSC/Cellartis group in combination with the microcavity array technology from the University of Leipzig group, we were able to monitor the contraction rate of all hCMCs over the whole duration of the experiment. It should be noted that each hCMC showed a superior beating stability over 45 days while all hCMCs were placed each or every second day on the microcavity array (MCA). The average relative change of the contraction rate for all clusters over 46 days was in the range of 2.5%. Such stability has never before been observed for a cardiomyocyte-based model and shows the great potential of the established hCMC model and the microcavity array technology.

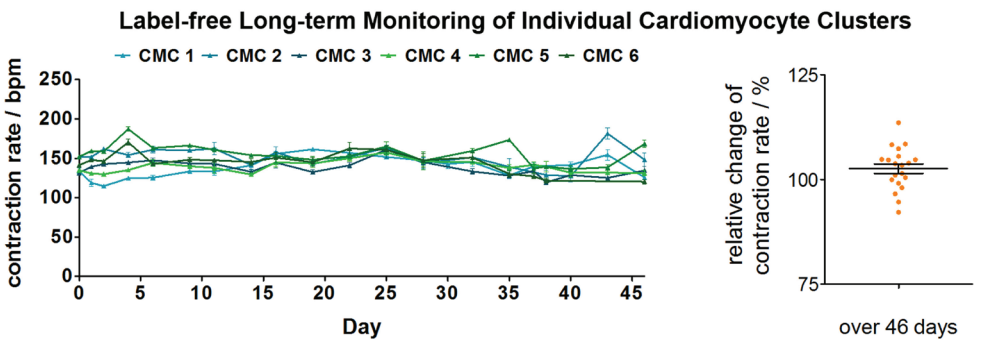


Figure 4.6 *The established microcavity-based long-term monitoring system in combination with the hES-derived cardiomyocyte clusters demonstrates the contraction rate stability over 45 days with a very small cluster-to-cluster variation and very small relative changes within each cluster over the whole monitoring time.*

Next we investigated the sensitivity of the hCMCs and monitoring system for adverse effects of reference compounds. First, sotalol was applied, a drug that causes QT prolongation but is not detected by classical hERG-channel screens. We detected the arrhythmia-causing effect of sotalol by analysing the concentration-dependent effect on the action potential duration, which was monitored by field potential recording (*Figure 4.7*, top panel). Furthermore, the pharmacologically relevant EC_{50} value was quantified (*Figure 4.7*, bottom left) and the concentration of 180 nM is in the range of clinically relevant concentrations (100–300 nM). We were also able to detect cardiotoxic effects such as early after depolarisation (EADs), which is a pathological event observed *in vivo* and causes life-threatening arrhythmias (*Figure 4.7*, bottom). Altogether, this demonstrates the high quality and sensitivity of the established hCMCs as well as the detection sensitivity of our label-free real-time monitoring system for adverse side effects of compounds such as arrhythmia.

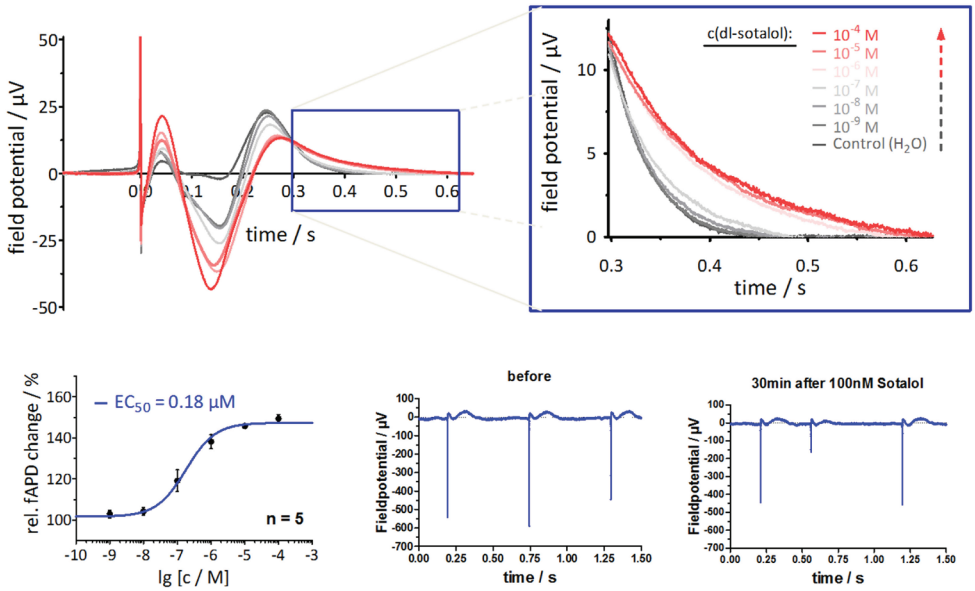
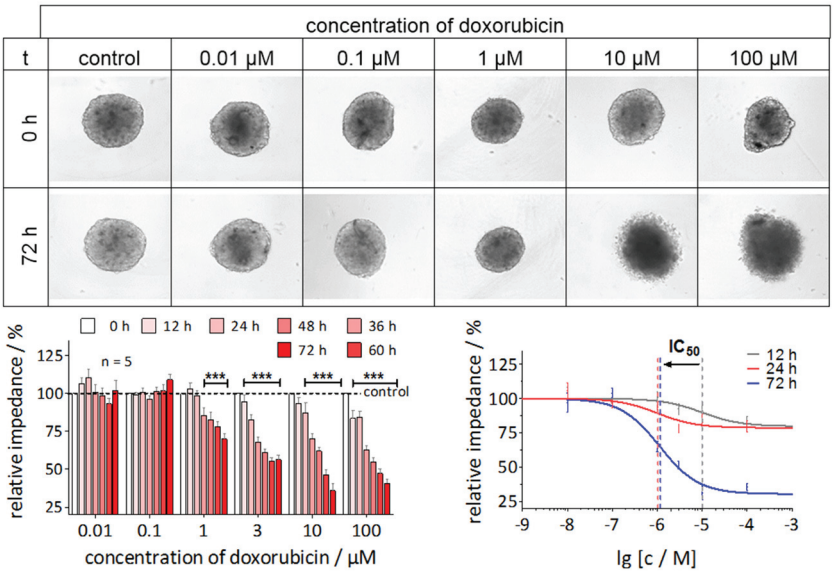


Figure 4.7 *Field potential recording allows the sensitive determination of adverse side effects of drugs as revealed by the prolongation of the action potential though sotalol treatment of hES-derived cardiomyocyte clusters. Pharmacologically relevant IC_{50} values were determined and pathophysiological in vivo effects, such as ventricular extra systoles, can be observed.*

Next, we used doxorubicin as a reference compound for the induction of structural cardiotoxicity. We incubated individual hCMCs with doxorubicin in the concentration range of 0.01–100 µM for 72 hours (*Figure 4.8*). No effects were observed based on the microscopic evaluation of each hCMC up to a doxorubicin concentration of 10 µM (*Figure 4.8,* top panel). In contrast, significant effects were detected using the impedance spectroscopy monitoring technique at 1 µM doxorubicin within 24 hours and for higher concentrations after just 12 hours.

The observed impedance decrease was correlated with a loss of cell integrity and degradation of subcellular structures. A time-dependent IC_{50} value was determined using the recorded spectra (*Figure 4.8*, bottom panel), which is in the range of 10 µM at 12 hours and around 1.1 µM at 24–72 hours of treatment.



time / h	12	24	36	48	60	72
IC50 / µM	9.88	1.01	1.05	1.18	1.29	1.13

Figure 4.8 *Impedance spectroscopy-based monitoring of adverse effects. To demonstrate bioelectronic label-free monitoring of toxic effects on hES-derived cardiomyocyte clusters, doxorubicin was applied in a concentration of 0.01–100 µM. The monitoring over 72 hours (microscopic images, top) illustrates a concentration-dependent decrease in the impedance-derived signal. Quantitative analysis allowed determination of* IC_{50} *values in a time-dependent manner on the same individual clusters.*

In summary, we demonstrated the high quality of the stem cell-derived cardiomyocyte clusters with regard to molecular and structural characteristics using functional readouts. In combination with our label-free bioelectronic monitoring system we proved the stability of hCMCs over weeks and, at the same time, the monitoring system is also capable of detecting and quantifying adverse effects of compounds. This system will now be used to perform extended repeated dose toxicity studies.

Bioelectronic monitoring of stem cell-derived neuronal networks: The monitoring of neuronal network activity by field potential recording is a commonly used technique for matured neuronal networks mostly obtained from primary cell preparations. The generation of active neuronal synaptic connections needs approximately 10–14 days *in vitro*, but the development

of subtypes of neuronal cells, as well as matured neuronal networks derived from stem cells as required for toxicity testing, is a lengthy process, and the stability of this system must be critically evaluated. To obtain neurons, for example from the forebrain, differentiation protocols have to start from pluripotent stem cells (hES/hiPS) (*Figure 4.9*, top). Early neuronal cell cultures are obtained with established protocols within 20 to 30 days. These cultures show high expression levels of neuronal markers such as b3-tubulin and Tau. Nevertheless, these neuronal cell cultures still reflect more an early embryonic stage than a matured network of neurons, and extended maturation of several weeks is necessary to for the development of a matured neuronal network (*Figure 4.9*, right top panel, 48 DIV).

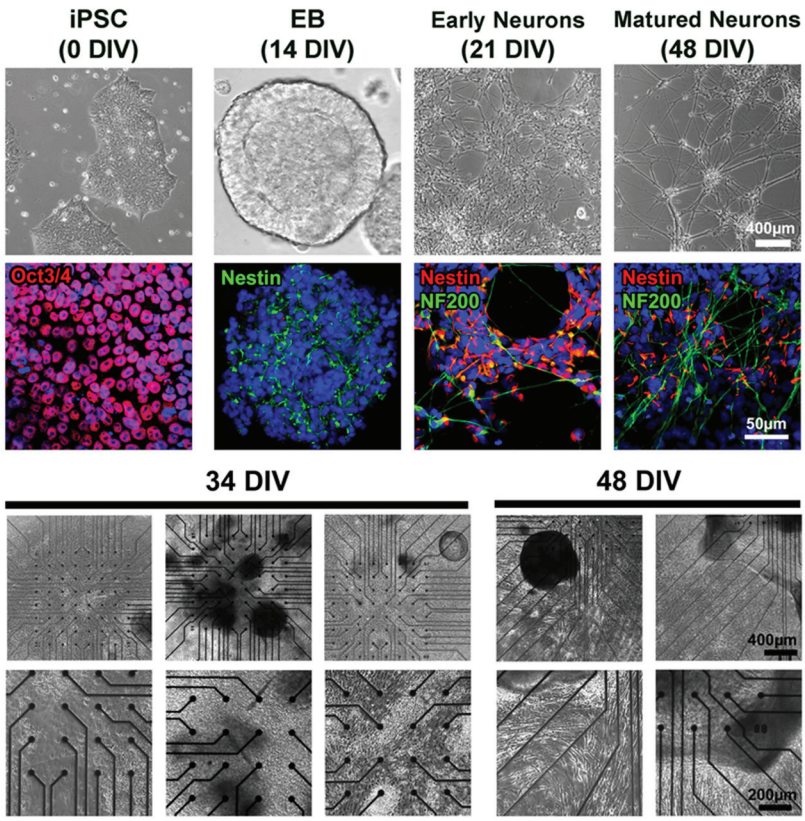


Figure 4.9 *Protocols of hES/iPS differentiation to matured neurons were optimised and adapted to a chip differentiation. In general, stem cells are growing in colonies (Oct3/4 – stem cell marker). The initiation of differentiation is achieved by generating embryonic body cultures (EB). Subsequently, matured neuronal networks are obtained by plating the EBs on coated dishes or microelectrode arrays. Long-term matured neuronal networks show a high expression of the axonal marker NF200 while the neuronal progenitor marker nestin diminishes.*

This was demonstrated by staining neuronal progenitor markers such as nestin, as well as markers of matured neurons such as NF200 and synapsin. The cultivation surface is critical for the differentiation and maturation process; since the cells undergo various alterations in adherence capabilities and cell-cell contact formation is lost, the cultivation surfaces are required for extended periods of time from five to seven weeks. A possible solution commonly applied to overcome this problem, is the use of surface coatings for optimised cell adhesion, which is potentially applicable in microelectrode arrays. In this case, clustering and detaching of the neurons from the electrodes complicate the reliable monitoring of matured neuronal networks and need to be taken into consideration. In this context, optimisation of the microelectrode array surface by modification of the passiviation layer of the arrays, as well as physicochemical modification of the electrode surface itself, is required. The University of Leipzig group adapted their bioelectronic equipment accordingly and observed an extended stability of neuronal networks over six to eight weeks (*Figure 4.9,* bottom panels). Highly active neuronal networks were reproducible when generated together with the ***SCR&Tox*** partners University of Bonn and Avantea and were monitored by means of the adapted high-density microelectrode array systems (*Figure 4.10*).

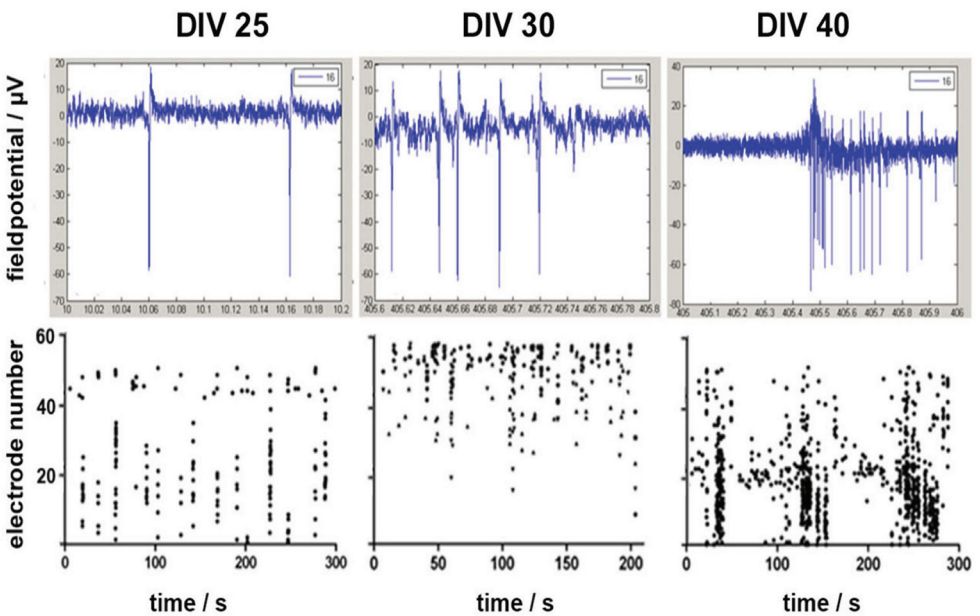


Figure 4.10 *Microelectrode array-based monitoring systems record the electrophysiological activity of neuronal networks. Maturation of neuronal networks leads to spontaneous electrophysiological activity. Extended maturation leads to series of action potentials (bursts – DIV 40). The analysis of the activity pattern can be used to identify adverse effects of compounds on neuronal network functionality.*

Alongside the detection and analysis of neuronal network activity as quality criteria for matured neurons, we monitored the maturation processes by impedance spectroscopy. From this data, early indications for a correct differentiation and maturation process can be obtained through the analysis of discrete alterations in specific frequency ranges. This provides the opportunity to identify aberrations in the long-term differentiation processes at an early stage and assures the quality of the stem cell-derived neuronal cell cultures.

Now that the opportunity to generate matured and electrophysiological active neuronal networks on optimised microelectrode arrays is available, we will use the system for toxicity testing and monitoring the induction and progression of neurotoxicity after exposure to standard reference compounds.

4.2.4 Innovation

Differentiation and maturation of hES/hiPS to specific cell types is being extensively developed and considerable improvements were achieved within the ***SCR&Tox*** project. Nevertheless, careful characterisation and validation of cell type-specific properties, as well as stability of these cells, must be done at each step. Especially with regard to the **SEURAT-1** objectives of replacing *in vivo* repeated dose toxicity testing with *in vitro* assays, unique cell type-specific functions have to be guaranteed to predict *in vivo* toxicity. In this context, we used our expertise on bioelectronic characterisation and monitoring of cell properties, cell function and (sub)cellular alterations. By optimising multielectrode array-based bioelectronic monitoring platforms according to the demands of stem cell-derived cells, the University of Leipzig group were able to produce novel microcavity arrays that are optimised for the human cardiomyocyte cluster (hCMC) produced by the CSC/Cellartis group within the ***SCR&Tox*** consortium. For the first time we could monitor the cardiac electrophysiological functionality within these hCMCs over more than 45 days. Moreover, in initial experiments reference compounds for arrhythmia as well as structural cardiotoxicity were successfully tested. Based on these results, the established system was proven to be fit for purpose and will now be used for the long-term monitoring of chronic/repeated dose toxicity effects.

Furthermore, in collaboration with the ***SCR&Tox*** partners University of Bonn and Avantea as experts on differentiation of neuronal cell cultures from hES/hiPs cells, we were able to overcome limitations in long-term cultivation procedures and establish highly matured and electrophysiologically active neuronal networks on our microelectrode array-based monitoring platform. This offers the opportunity for the detailed analysis of specific neurotoxicity by functional bioelectronic monitoring over days or even weeks. We will use the established system together with the neuronal groups within the ***SCR&Tox*** consortium to investigate and validate the generated neuronal cell culture models with selected reference compounds.

4.2.5 Cross-Cluster Cooperation

The fact that some ***SCR&Tox*** partners participate in other consortia from the **SEURAT-1** Research Initiative fosters collaboration between different projects (for example, the Karolinska Institute is also involved in NOTOX; the Joint Research Centre is a partner in DETECTIVE; and the UKSCB participates in ToxBank).

Furthermore, ***SCR&Tox*** members are leading the cross-cluster Stem Cell Working Group and meets on a regular basis by teleconference (see section 4.10.7). ***SCR&Tox*** has actively participated in the joint workshop on Bioreactors and Cell Engineering organised by *HeMiBio* (a workshop summary report is given in section 4.10.9) and follow-up discussions were held at the **SEURAT-1** Annual Meeting in March 2013 in Lisbon. A follow-up workshop will be organised for September 2013. ***SCR&Tox*** and DETECTIVE organised a joint meeting on repeated dose toxicity protocols in heart models, which was held in May 2013 via web-conference. The objective of this meeting was to share methodologies and data and thus avoid doubling-up of efforts.

SCR&Tox has made a proposal to start discussions on the most interesting assay for one toxicity pathway on which particular focus will be placed in the second half of the work programme (see section 4.10.9). To do this, ***SCR&Tox*** has coupled a regular project meeting in Ispra, Italy, with a half-day meeting entitled 'Assay Development Preparatory Meeting' with members of COACH.

4.2.6 Expected Progress within the Third Year

Biological resources are now available as standard products both at the undifferentiated stage – pluripotent stem cell lines of embryonic origin or induced by non-integrative gene transfer – and for many lineages at different stages of differentiation. Large-scale production on the one hand, and miniaturisation of the culture format on the other, are now completing against the bank technologies developed to ensure optimal transfer to industrial platforms. In parallel, several cell models have been developed, involving preliminary assays of chronic drug toxicity that look very promising, and a major effort has successfully implemented new functional testing of cells challenged with chemicals, in particular for excitable ones, neurons, cardiomyocytes and muscle.

According to the work programme ***SCR&Tox*** will, from now, focus on two perfectly integrated activities: assay development, and implementation on an industrial-scale platform of one assay selected as a proof of concept. This year will be used for selecting the most promising assays to be developed, both internally at ***SCR&Tox*** and together with all the other **SEURAT-1** consortia that have been called to participate in discussions and to provide their own tools. This selection process will culminate at the beginning of September 2013, when the programme for the second period of the network will be officially finalised.

4.2.7 Future Perspectives

The capacity to generate and differentiate patient-derived induced pluripotent stem cells (iPSC) into many relevant cell types, and their amenability to genetic engineering, opens up the possibility of systematic industrial iPSC banking and differentiation to provide cells or tissues recapitulating human genetic diversity, physiology and pathology. However, it may be difficult to recapitulate the phenotype of complex and multifactorial diseases or toxic responses in isolated cells. To solve this problem, a '*genome-based combinatorial approach for drug discovery and predictive toxicology testing*' was envisaged. This new paradigm relies on the four main attributes of iPSCs that make them a most promising tool:

- Their indefinite self-renewal capacity at the undifferentiated stage, allowing provision of any amount of cells with a common genetic background, as well as consistency in biological material.
- Their pluripotency. Depending on the identification of appropriate and robust protocols, this allows differentiation into any cell phenotype of interest with a common genetic background.
- Their potential at expressing any human genomic background. Given that suitable donors are available, iPSC lines can provide a cell model for any human genotype.
- Their amenability to genetic engineering, allowing the generation of discrete models of gain or loss of gene function in any cell phenotype with an otherwise common genetic background.

These properties have already permitted both the successful identification of molecular mechanisms associated with monogenic diseases in iPSC progeny and on-going studies aiming at using these *in vitro* models for high-throughput screening in drug discovery to identify safe drug candidates. This approach will open new paths for predictive toxicology. We hypothesise that toxic responses in target organs from patients are different from those responses in healthy individuals, and, therefore, safety testing of new drugs should be fitted to iPSC lines of the relevant clinical population. Large banks of iPSC lines derived from randomly sampled specific patient groups, and from supposedly healthy people as a reference, both also representative of human genetic diversity, will allow us to establish predictive target-population-specific toxicology screens to challenge drugs while still at a pre-clinical development stage. Emphasis will be on the development of relevant 3D models using an appropriate combination of cells mimicking the *in vivo* toxicity. Such molecular screens could be used for direct comparison of toxicology profiles, benchmarking drug candidates with existing molecules and enabling a 'phase III study in a dish'. Furthermore, where some drugs are toxic to certain patient subpopulations, which may be due to their (epi-)genetic 'polymorphisms', iPSC lines provide a basis for 'population scale' analyses seeking discrete

polymorphisms involved in an observed toxic phenomenon and, by extension, the molecular pathways that may be affected by the change in gene expression or function related to those polymorphisms. Most importantly, that knowledge of affected molecular pathways may lead to novel toxicity testing strategies and assays. We thus envisage *a genome-based combinatorial approach for predictive toxicity* involving stratified cohorts of patients treated with the same compounds but which exhibit differential toxicity profiles. The genomic and epigenomic alterations critical for the toxicity will thus be identified and the pathways analysed using transcriptomics and proteomics.

Predictive biomarkers could be investigated in subpopulations of patients who exhibit toxic responses to drugs by using different sources of iPSC lines. As a first hypothesis: safety testing of new drugs should be fitted to iPSC lines of the relevant clinical population, since toxic responses in patients are different from those in healthy individuals. Therefore, large banks of iPSC lines derived from randomly sampled specific patient groups could be used to establish predictive target-population-specific toxicology screens to challenge drugs in the relevant clinical population, in comparison to healthy controls. Different cell progenies deemed potential targets for organ toxicity will be used to determine a toxicity profile of the drug using a standard pre-determined set of measures exploring cell functions, among others those provided by ***SCR&Tox*** and other projects of the **SEURAT-1** Research Initiative. Furthermore, the iPSC-derived models could be used to develop new predictive mechanism- and organ-specific screens based on integrated cross-omics studies to identify the most robust and conserved pathways. The main advantage of iPSC lines, within that framework, is the amenability they offer to seek so-called 'pathways of toxicity'; i.e., signalling pathways that are discretely altered by the toxicant in the cells replicating a specific phenotype of interest. It is also important to underline that chronic, rather than acute, toxicity associated with repeated dosing is most often the problem when drugs are already on the market, as these have successfully gone through usual toxicity tests. Relevant derivatives and combinations of iPSC lines in 2D and 3D formats could be used to design paradigms based on long-term cell cultures repeatedly treated with sub-acute toxic doses, which may provide identifying signalling pathways discretely affected by such prolonged treatments with no conspicuous acute toxic effects.

As a second hypothesis: toxicity of a drug in a subpopulation of patients is influenced by gene polymorphisms that discretely affect specific cellular mechanisms. In this setup, toxic-responders and non-responders from cohorts of treated patients could be used to search for differential impact on cellular responses. If the drug affects differential signalling pathways in cells derived from the two groups of patients (i.e., identified toxic-responders versus non-responders), the experimental paradigm will explore those systems in a combinatorial fashion, in a search for the candidate genes most likely responsible for those differences. Efforts could be made to incorporate iPSC-derived immune cells into the systems to include immune-mediated reactions. Associated biomarkers will be sought, the identification of which may help to develop predictive tools for screening drug safety.

These strategies are summarised in *Figure 4.11*.

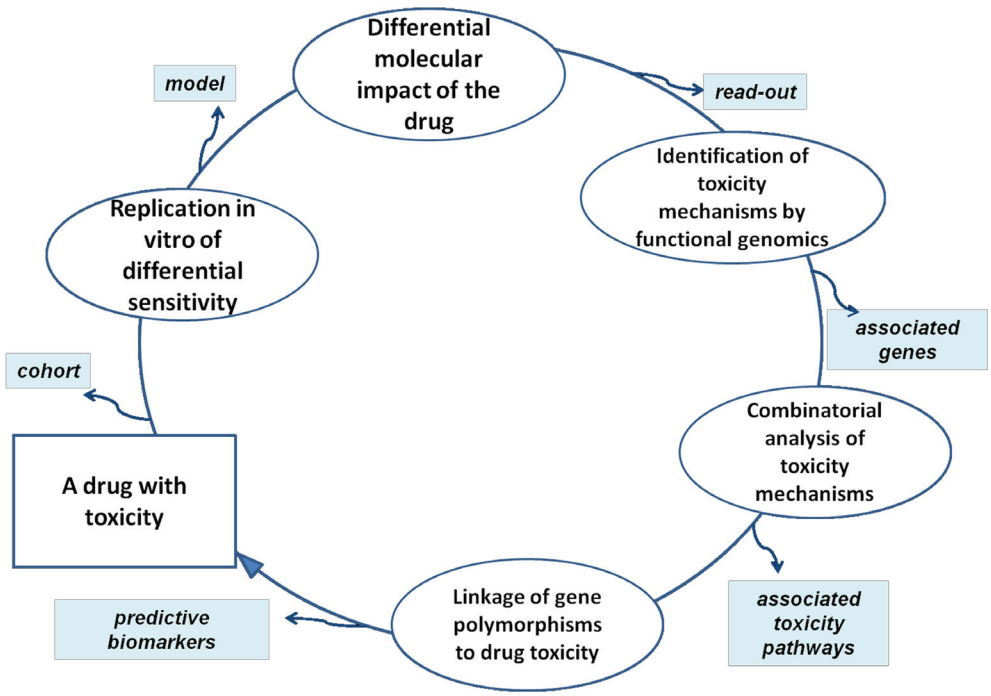


Figure 4.11 *Outline of an innovative approach for harnessing pluripotent stem cells for toxicology.*

These approaches could be a natural consequence of the ***SCR&Tox*** programme for the development of a research strategy to replace animal testing in the safety evaluation stage, and could also be relevant for the planning of a possible SEURAT-2 project cluster.

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4.3 HeMiBio: Hepatic Microfluidic Bioreactor

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4.3.1 Introduction and Objectives

Refinement, Reduction and Replacement of animal usage in toxicity tests, the so-called 3Rs principle, is of particular importance for the implementation of relevant EU policies, such as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation (EC1907/2007) or the 7th amendment to the Cosmetics Directive (76/768/EEC). Although multiple projects funded by the European Commission have aimed to implement the 3Rs principle in toxicity testing, the assessment of toxic effects of chronic exposure still requires a high consumption of animals. Aside from these ethical considerations, there is a great need for suitable human cells for toxicity testing, due to the poor concordance between human and animal models.

In ***HeMiBio***, we propose to *generate a liver-simulating device mimicking the complex structure and function of the human liver*. The device will reproduce the interactions between hepatocytes and non-parenchymal liver cells (hepatic stellate, sinusoidal endothelial and Kupffer cells) for over one month *in vitro*. Such a Hepatic Microfluidic Bioreactor could serve to test the effects of repeated exposure to chemicals, including cosmetic ingredients. To create such a device, the cellular components of the liver need to be viable for over one month, with *in vivo*-like metabolic and transport function and physiology. The latter includes (i) flow through the device; (ii) zonation of the hepatocytes (and some non-parenchymal liver cells); and (iii) impact of the non-parenchymal cells on the function and downstream toxicity of hepatocytes. The device should be able to (iv) screen drug-drug interactions as well as long-term toxicity of chemical entities. Finally, (v) the effect of enzyme inducers and inhibitors on the function of the liver-simulating system should be testable. However, no bioreactor has yet been created that can indeed fulfil all criteria set forth above. With increasing complexity, hepatocyte function is maintained over extended periods of time, whereas the less-complex culture systems are more amenable for studying the mechanisms that control maintenance of cellular function.

As human livers, from which the different cellular components could be selected, are in general unavailable for studies in the cosmetic and pharmaceutical industry, due to liver donor shortage, we propose to isolate the cellular components from differentiated pluripotent cells. Pluripotent cells are normally derived from the blastocyst, as embryonic stem cells (ESCs). Alternatively, they can be created from mature terminally differentiated cells by introduction of pluripotency genes, which leads to the generation of induced pluripotent stem cells (iPSCs). One of the ***HeMiBio*** partners has shown that ESCs and iPSCs can differentiate to immature hepatocytes, as well as cells with liver sinusoidal endothelial cell- (LSEC) and hepatic stellate cell- (HSC) features, which will be used to generate the liver-stimulating device. We also believe that creation of the device will aid in inducing further maturation of these three cellular components. As an alternative we will test whether cells isolated from livers can be expanded by genetic manipulation using the UpCyte® technology, without loss of mature cellular function.

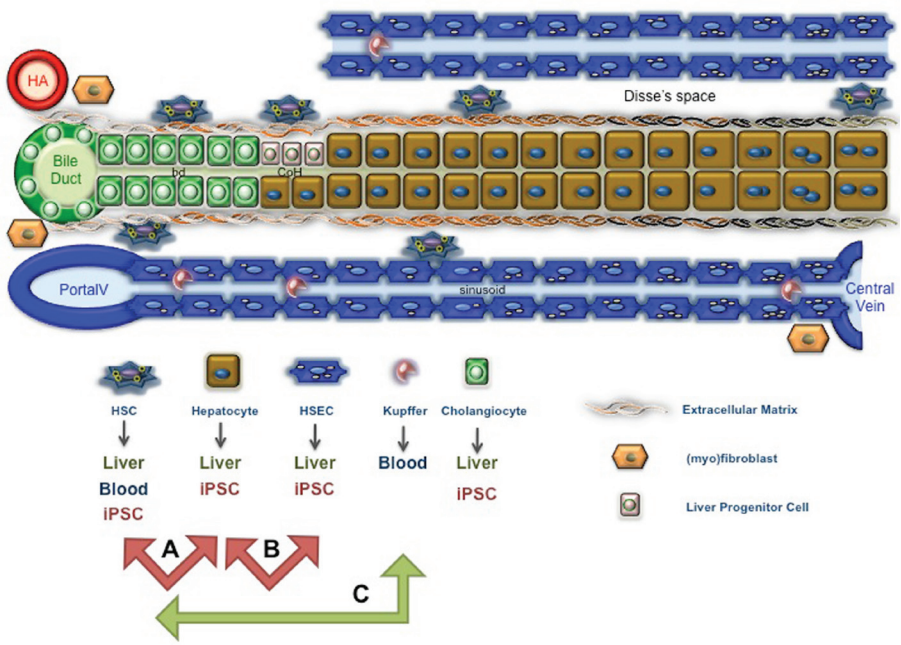

Figure 4.12 *Schematic representation of a liver sinusoid (adapted from: Dollé et al., 2010).*

The underlying hypothesis for the successful creation of a 3D liver-simulating device suitable to test repeated dose toxicity is that: (i) *hepatocytes* and *non-parenchymal cells* need to be combined; (ii) both *homotypic and heterotypic cellular interactions between the different components are required* to maintain the functional, differentiated and quiescent state of each cell component; (iii) (a) the *matrix* whereupon cells are maintained, (b) *oxygenation*, and (c)

nutrient transport will need to be optimised to support long-term maintenance of hepatocyte and non-parenchymal cell function, in an environment where shear forces are kept at their *in vivo*-like levels; (iv) the system needs to be built such that *repeated online assessment* of cellular integrity, as well as metabolic and transport function, and physiology of the different cellular components, is possible.

Although the exact configuration as shown in *Figure 4.12* may not be required, the very short-distance cellular interactions shown between (A) hepatocytes-LSEC and (B) hepatocytes-HSC cells will be required for maintaining the functional state of the three cell types, and (C) the presence of monocytes/Kupffer cells will be required to fully assess drug toxicity.

To achieve the creation of a liver-bioreactor taking into account the hypotheses stated above, the specific objectives are:

- to develop tools to engineer *three different liver cell types* (hepatocytes, LSECs and HSCs) generated from iPSCs, or from primary cells, expanded using the UpCyte® technology;
- to incorporate *molecular sensors* and *electro-chemical sensors*, to allow assessment of function and cell integrity;
- to develop a *2D-bioreactor* to evaluate the role of cell-cell and cell-matrix interactions in the maturation and maintenance of functional hepatocyte and non-parenchymal cells. The platform will serve as a rapid intermediary to the 3D-bioreactor, and be used to explore varying sensor designs and cell interactions needed in the more complex design;
- to generate a *3D liver-simulating device* by combining the above-mentioned engineered cells and sensors, which will allow dynamic monitoring of cellular function and health;
- to provide proof-of-principle that a liver-simulating device can *recreate the toxicity profile in vitro* of toxins with a known *in vivo* toxicity profile over a minimum of one month;
- to assess the *molecular, functional and metabolic phenotype* of the hepatocellular, LSEC and HSC components at all stages of bioreactor development, and compare this with that of cells freshly isolated from human livers.

4.3.2 Main Achievements and Challenges in the Second Year

During the last year, extensive focus was on (i) optimisation of the cell-culturing methods and subsequent characterisation; (ii) the generation of molecular sensors; (iii) the improvement

and generation of microsensors; (iv) the optimisation of the bioreactor design.

The achievements and challenges at a glance are:

Fresh cells as controls:

- Culture optimisation of HSCs and LSECs on-going, not yet fully characterised;
- Characterisation of fresh LSEC, HSC and 'zonated' hepatocytes: '-omics' being evaluated.

PSC-derived hepatocytes:

- 3D cultures improve hepatocyte differentiation, but need further evaluation to determine how close they are to primary hepatocytes;
- Knock-in add-on homologous recombination is possible using zinc fingers, in DNA regions that are 'closed' in iPSCs.

Upcyte® hepatocytes:

- Further characterised, demonstrating good metabolism and apparent reliable toxicological characteristics;
- Would be useful to compare with HepaRG cells.

Molecular sensors:

- Generated master PSC cell lines for recombinase-mediated cassette exchange in the AAVS1 locus of toxicity cassettes;
- Cloning of all three reporters (caspase-7, NF-kB, p53).

External sensors (pH, O_2, glucose, lactate):

- Characterisation finalised;
- Automated routine for monitoring of supernatant assessed;
- Integration of O_2 sensor with 2D-bioreactor started.

ALT (glutamate):

- Target detection limit and sensitivity achieved.

NH_4^+/urea:

- Stable sensors solution identified.

Bioreactor design:

- Cell capture device functional;
- 2D-bioreactor (flow-over) allows maintenance (short term) of Upcyte hepatocytes;
- Production of a microfluidic switchboard enabling automation of fluid sampling.

4.3.3 Characterisation of the Phenotype of Cells to be Incorporated in the Bioreactor

Introduction

As ***HeMiBio*** wishes to create a microbioreactor containing not only the parenchymal hepatocytes but also non-parenchymal cells, such as liver sinusoidal endothelial cells (LSECs) and hepatic stellate cells (HSCs), it was required that LSECs and HSCs from human origin be characterised. Accordingly, we performed transcriptome, methylome and miRNA analysis of freshly isolated LSECs and HSCs during the last year, as well as from a subset of these cell populations (HSCs), after activation in culture.

As Upcyte® liver cells originate from native primary liver cells, it was mandatory to verify that the Upcyte® liver cells carried the signature structures and functions of hepatocytes, LSECs and HSCs.

Approach

Upcyte® cells: Human liver resections were obtained from the National Hospital, Oslo, from surgery on patients with hepatic secondary colorectal carcinoma tumours. Following shipment through a specially arranged rapid air transportation from Oslo to Tromsø (Vascular Biology Research Group, University of Tromsø) the resections were perfused with collagenase through all visible veins, and the resulting single cell suspension was subjected to velocity and density centrifugations in Percoll gradients to produce purified suspensions of hepatocytes and non-parenchymal cells. Freshly isolated hepatocytes were immediately frozen down in aliquots. Viability and purity were assessed for each donor. Frozen pellets of hepatocytes were shipped to ***HeMiBio*** partner Medicyte for the Upcyte® technology. The non-parenchymal cell fraction was seeded overnight and washed free of non-attached cells and debris the following morning. Liver sinusoidal endothelial cells (LSECs) were isolated and purified by fluorescence-activated cell sorting after incubation with specific endocytosis markers prepared in-house in the Vascular Biology Research Group, University of Tromsø. The purified LSECs were frozen down and shipped to Medicyte, where the Upcyte® technology was applied after a short incubation and initial proliferation.

Methylation profiling by MeDIP-chip: Specifically, the DNA methylation profile of all Refseq promoters was identified by methyl-DNA immunoprecipitation (MeDIP)-chip for hepatocytes (Hep), LSECs, quiescent (uncultured) HSCs and activated (cultured) HSCs. Cells from several donors were examined. MeDIP-Chip was done in duplicate for most of the above-mentioned cell types. For cell types for which too little material was obtained, one MeDIP replicate was done.

Results

Upcyte® hepatocytes: Upcyte® hepatocytes generated from different donors have been characterised further, mainly in two different laboratories, with respect to their functionality and response to hepatotoxic chemicals from the ToxBank list and non-hepatotoxic compounds in 2D culture. Upcyte® hepatocytes derived from different donors differed only slightly in their response to the compounds. All compounds could be correctly classified as non-toxic, moderate and toxic compounds, which is in accordance to *in vitro* data from primary hepatocytes. There is a good correlation between the IC50 values in primary and Upcyte® hepatocytes (data not shown, publication in preparation).

Phase I metabolic activities, mRNA expression and induction responses to drugs are similar to primary cultured hepatocytes. Likewise, transporters are expressed to a similar level and bile canaliculi could be successfully stained using CDF, suggesting functional MRP2 transporter activity. Polarisation was also demonstrated using specific markers for apical and basolateral compartments. Phase II activities were similar to freshly isolated hepatocytes (data not shown, publication in preparation).

Initial studies on ZFN-mediated integration of a bioreporter construct showed that integration is possible but single cell cloning has not been successful so far.

Upcyte® LSECs: Freshly isolated human LSECs from several donors have been characterised using scanning electron microscopy, immunocytochemistry, fluorescence microscopy and RT-qPCR (carried out by ***HeMiBio*** partner KU Leuven). The cells express the unique fenestration, tissue-specific profile of scavenger receptors, and maintain the LSEC signature function, namely receptor-mediated endocytosis. Four of seven primary LSEC populations were successfully treated with Upcyte® protocols. Cells could be grown to PD20 for donor 2, PD19 for donor 3, PD48 for donor 6 and PD35 for donor 7 before the cells went into senescence. Growth curves were monitored and initial characterisation was performed demonstrating that LSEC markers are present. Two donor samples are currently in production to working cell-bank stage to provide cells to ***HeMiBio*** partners.

Upcyte® HSCs: Attempts to treat freshly isolated human quiescent HSCs with Upcyte® protocols have not been successful, as they acquire an activated status. We have, therefore, focused on the optimisation of culture conditions that can transform the cultured-activated HSCs into a more quiescent phenotype. These inactivated HSCs should still be responsive to fibrogenic cues, allowing for the screening of pro-fibrotic compounds *in vitro*. Once fully optimised we can use these conditions to inactivate HSCs, which were previously activated using Upcyted® protocols.

Methylation profiling by MeDIP chip: Replicate data show robust correlations between replicates ($0.86 < r < 0.96$), indicating high reproducibility. Dynamic range of log2 MeDIP/input ratios is -1 to +1.5, consistent with our earlier data (*Figure 4.13*).

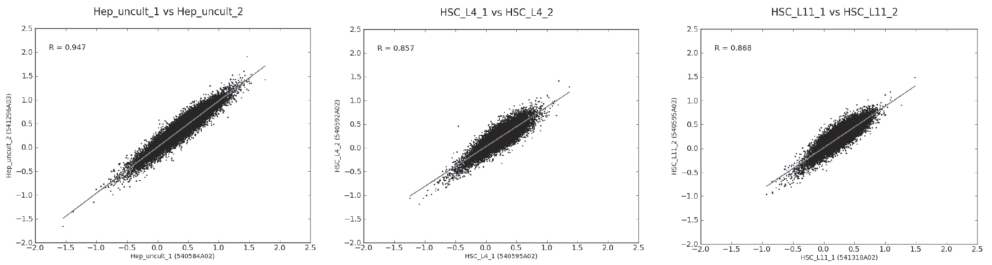

Figure 4.13 *Samples of replicate plot analysis of MeDIP-chip replicates. The rest of the samples show similar profiles and correlations (not shown).*

The number of genes with a methylated promoter (determined by identification of methylation peaks, KS test P<0.001) ranges from ~4800 to ~8000 for all cell types (*Figure 4.14*). The lowest numbers are seen for cultured cells (aHSCs), suggesting culture-induced demethylation events. This is however being examined further, taking into account the numbers of replicates considered in the analyses.

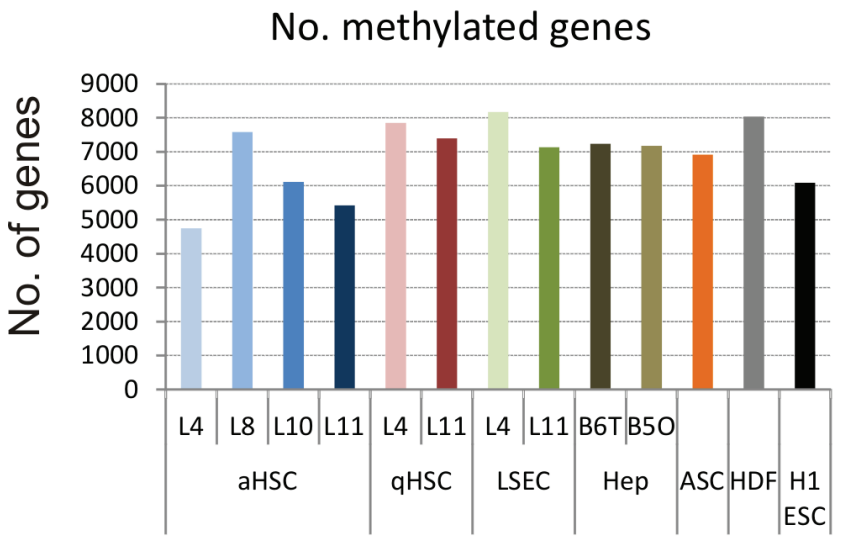

Figure 4.14 *Number of the methylated genes identified by MeDIP-chip for the liver cell types examined. Data for adipose stem cells (ASC), fibroblasts (HDF) and H1 ESCs are also shown.*

Comparison between uncultured liver cell types: qHSCs, LSECs, Hepatocytes: A two-way comparison between all cell types shows overall 70–80% similarity in promoter methylation. A three-way comparison between uncultured cell liver types (hepatocytes, LSECs and qHSCs) identifies a 'methylation core' of 3541 genes, leaving 700–800 genes uniquely methylated in these cell types (*Figure 4.15*). Gene ontology (GO) terms associated with these genes mainly include genes encoding proteins with reproduction and germline functions, indicating long-term repression of these functions by DNA methylation in these adult cell types. GO terms enriched for Hep-, LSEC- and qHSC-specific methylated genes reveal distinct, specialised functions between these cell types.

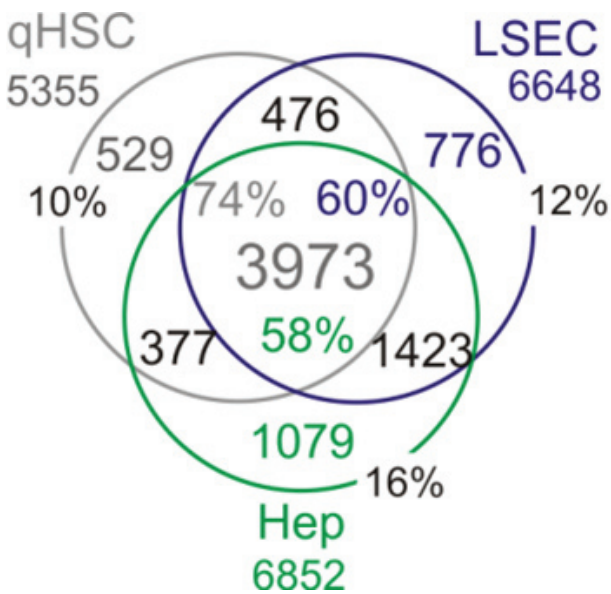

Figure 4.15 *3-way comparison of DNA methylated genes between uncultured liver cell types.*

Methylation of HSCs: A two-way comparison of methylated genes between all donors for activated HSCs (two replicates for all samples) shows that donor L8 is most similar to all other donors while donor L4 is the least similar (*Figure 4.16*, left). This likely results from L8 and L4 having the highest and lowest, respectively, number of methylated genes (see *Figure 4.14*). A four-way comparison between aHSC donors shows a core of 2272 methylated genes, making up only ~30% to ~50% of all methylated genes in these donors (*Figure 4.16*, right). This indicates large epigenetic variation between donors upon culture of HSCs. Comparing activated HSCs or quiescent HSCs for L4 and L11 indicates greater similarity between donors.

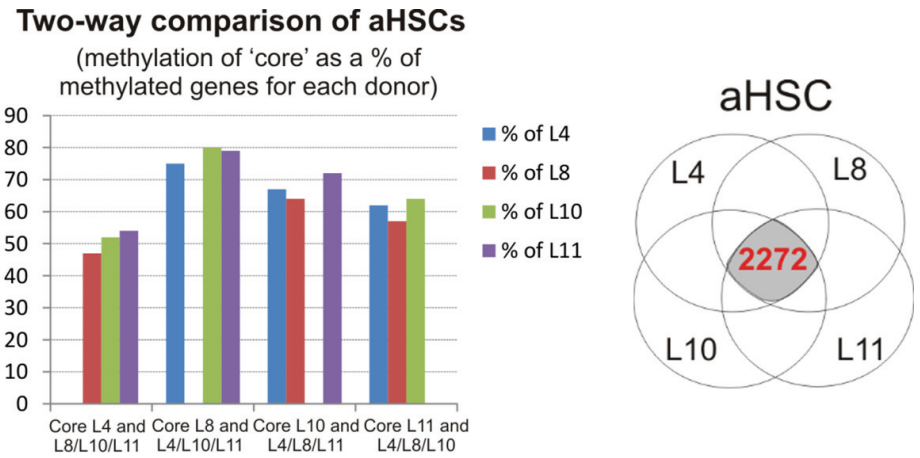

Figure 4.16 *Two-way comparison of methylated genes among activated HSCs (aHSCs; left) and four-way Venn diagram analysis of methylation among the four aHSC donors (two replicates per donor; right).*

A study of quiescent versus activated HSCs is being finalised. Examples of promoters showing similar and different methylation patterns between quiescent and activated states are shown in *Figure 4.17*.

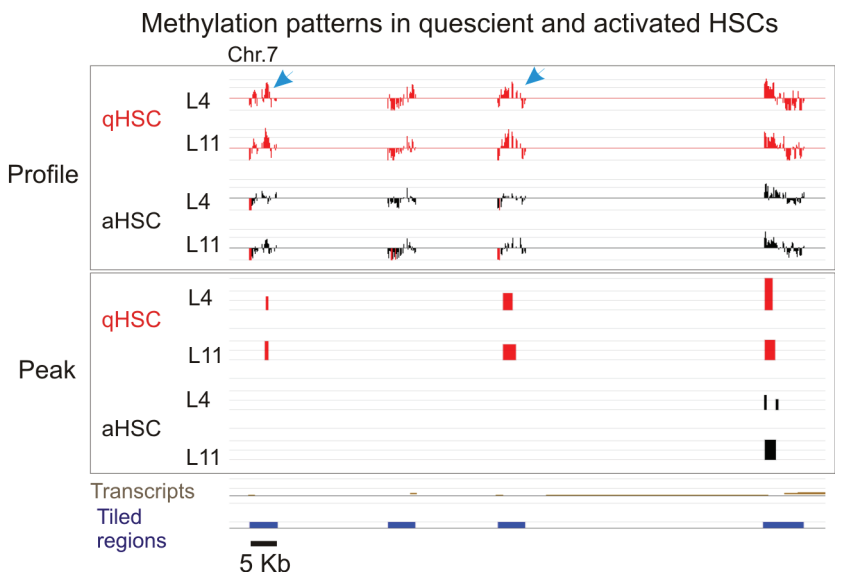

Figure 4.17 *Browser view of methylation profiles and peaks in a stretch of chromosome 7 in quiescent HSCs (qHSCs) and activated HSCs (aHSCs).*

Methylation of uncultured cell types: Comparison of LSECs and quiescent HSCs (two uncultured cell types) reveals 70–80% similarity in methylation pattern between these cell types (*Figure 4.18a*). Hepatocytes from two donors also show 80% similarity with a core of 5816 methylated genes (*Figure 4.18b*). We also noted the high number of methylated genes in hepatocytes.

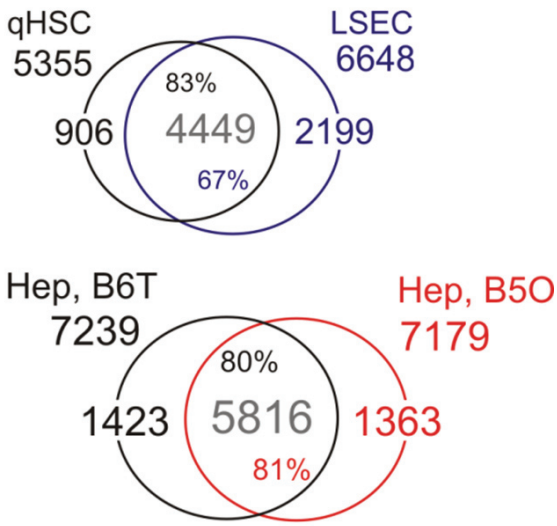

Figure 4.18 *Venn diagram analysis of methylation in uncultured cell types: a) methylation core of quiescent HSCs L4+L1 and core LSECs L4+L11; b) donor comparison of hepatocyte methylation.*

4.3.4 Sensors

Introduction and Approach

Molecular sensors: In ***HeMiBio***, Zinc finger nuclease-mediated homologous recombination (ZFN-HR) is being used to insert selection cassettes downstream of either a gene expressed specifically in mature hepatocytes, HSCs or HSECs, or cell damage-specific expression cassettes (NF-κB, p53 and caspase-3). We hypothesise that this combination allows precise detection of toxic effects on any of the three cell components to be incorporated in the bioreactor. It was shown that gene targeting in human iPS cells can be achieved at high frequency by using ZFN and cleverly designed targeting/selection cassettes (*Hockemeyer et al., 2009*). In the second **SEURAT-1** Annual Report we demonstrated that we have successfully inserted Flippase Recognition Target sites into *AAVS1* (Adeno-associated virus integration site 1) using optimised ZFN nucleases in hPSCs.

External sensors: The ability to fabricate complex microfluidic systems with compatible dimensions between the microfluidics and biological cells has recently attracted significant attention in the development of microsensors for analysing online and in real-time biophysical and biochemical functions of cells (*Yi et al., 2006; Yang et al., 2008*). In contrast to state-of-the-art optical readout systems, integrated microsensors appear better suited for long-term monitoring of cultured cells and tissues. In addition, they can be located almost anywhere in complex 3D-cell culture systems, allowing measurement even in optically hidden areas. Furthermore, standard optical read-out systems often provide end-point detection results, rather than continuous measurement revealing complementary sequences of information. The development of miniaturised detection modules with high sensitivities and signal-to-noise ratios, and fast response times is therefore of utmost importance. Among the most common detection methods used so far, optical and electrochemical detection techniques are the most frequently employed.

As reported in the second **SEURAT-1** Annual Report, we selected sensors for general cell-culture monitoring in the first phase of the project. They have been fully characterised and a routine for the monitoring of cell culture supernatant has been assessed.

Results

Molecular sensors: We generated master PSC lines suitable for recombinase-mediated cassette exchange (RMCE). An FRT-flanked cassette containing the CAGGS-promoter eGFP-P2A-hygromycin^R-thymidine kinase cassette was introduced in the AAVS1 locus, located in the PPP1R212C gene, using Zinc finger nuclease-mediated homologous recombination (ZFN-HR) in hESCs and hiPSCs. Using flippase, we can exchange the sequence between the FRT sites with a cassette containing the Puromycin^R gene, as well as a promoter for a gene expressed at a specific step of hepatocyte differentiation that drives tdTomato-T2A-Hygromycin^R expression. Double positive (Puro^R) and negative (Gancyclovir^R) selection allows for the generation of uniform single-copy promoter-containing cells in ±15 days. Likewise, we can use flippase-mediated exchange of the cassette with a cassette including molecular sensors for instance cell apoptosis (caspase-7), oxidative stress (Nrf2) or other cellular toxicity events.

External sensors: Sensors for pH, glucose, lactate (electrochemical) and O_2 (optical) have been characterised using an automated setup to withdraw the sample directly from a cell culture flask. The developed sensing protocol shows sensitivity and stability compliant with the requirements of *in vitro* cell-culture monitoring. The calibration in temporal proximity to the actual measurement provides the means to easily compensate for any drift that may occur. An example of lactate measurement is given in *Figure 4.19*.

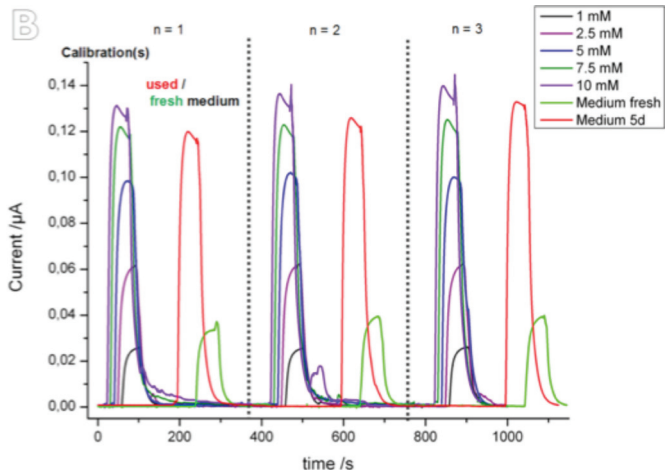

Figure 4.19 *Example of repetitive lactate measurements in cell culture medium, showing the reproducibility of calibration (left peaks) and analysis of fresh medium or medium drawn from a five-day-old cell culture (red and green curves).*

Sensors for cell culture health monitoring, namely urea/NH_4^+ and ALT sensors, have been fabricated and the characterisation in buffer solutions showed characteristics adapted to the target measurements. Establishment of a measurement protocol, adapted to *in vitro* conditions and automation, is in progress.

The fully characterised sensors have been integrated with the 2D cell capture device (see below; schematic shown in *Figure 4.20*). Sensors for pH, lactate and glucose, and in the future urea/NH_4^+ and ALT, are located downstream from the cell culture chamber, in the sensor module (pink). Optical O_2 sensors are located at the inlets and outlets of the cell culture chamber, allowing monitoring of the oxygen consumption in the chamber.

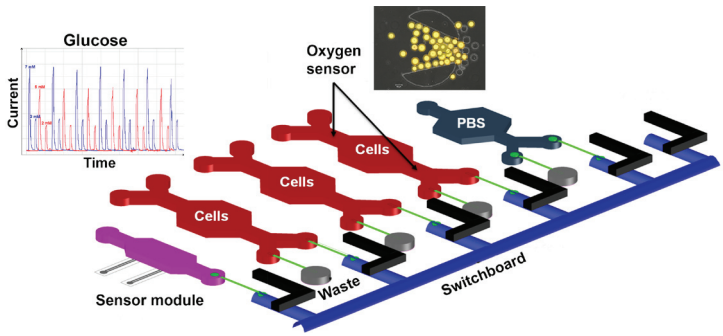

Figure 4.20 *Schematic diagram showing integration of sensors with the 2D cell culture capture device.*

4.3.5 Bioreactor Design

Introduction

Bioartificial liver devices are still under scientific development, but are currently effectively used in a clinical context with the purpose of replacing hepatic function in patients with liver failure (*Kobayashi, 2009*). Their use for toxicity screening of new chemical entities is in its infancy, but small-scale laboratory systems based on human cells are believed to be very promising for a variety of research purposes, including investigations on xenobiotic metabolism, hepatotoxicity, liver function and liver disease (*Dash et al., 2009*).

Approach

We are generating evermore complex bioreactors to culture hepatocytes, stellate and endothelial cells for >28 days. We hypothesise that this will lead to further maturation of immature cells derived from iPS cells and assure their persistent differentiated and quiescent state for lengthy periods of time. The used materials, as well as first designs of the bioreactors to be used for cell culturing and toxicity testing, were reported in the second **SEURAT-1** Annual Report (*Sancho-Bru et al., 2012*). In the second year we tested whether (immature) hepatocytes, LSECs and HSCs can be captured from mixed iPS cells cultures by microfluidic isolation on hepatocyte-, LSEC- and HSC-specific antibody-micropatterned surfaces. In addition, we have further developed the 2D-bioreactor and integrated external sensors as described above.

Results

We created a 2D cell capture device (*Figure 4.21*) and demonstrated that it is functional; i.e., endothelial cells and hepatocytes (all cell lines) can be immobilised on the capture device with almost 100% purity and a yield of 20% (*Figure 4.22*).

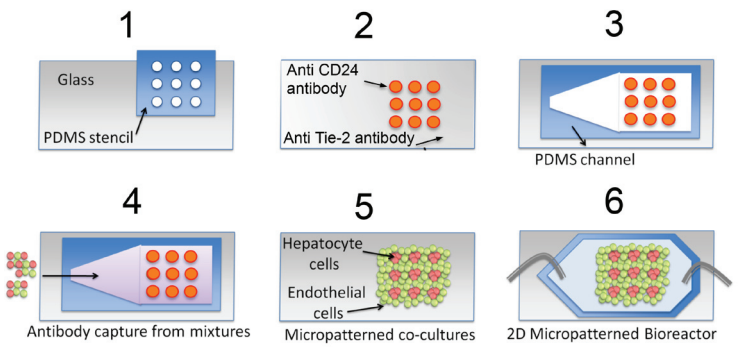

Figure 4.21 *Construction of the cell capture device.*

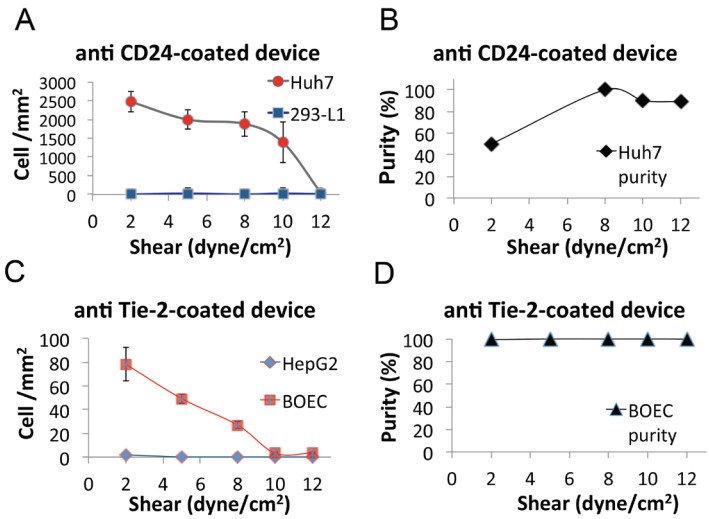

Figure 4.22 *Isolation of hepatocyte and endothelial cell lines on the cell capture device.*

We have successfully developed a 2D- (flow-over) bioreactor (*Figure 4.23*) and demonstrated that the device allows the short-term maintenance of Upcyte® hepatocytes. The device also allows for integrated on-chip measurement of oxygen uptake rates.

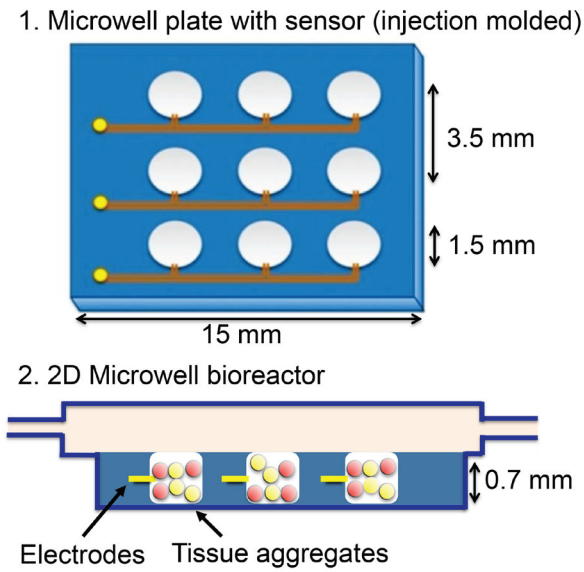

Figure 4.23 *Design of the flow-over device.*

4.3.6 Cross-Cluster Cooperation

On the occasion of the 18-monthly ***HeMiBio*** meeting in Gent, Belgium, on 25–26 June 2013, ***HeMiBio*** invited investigators from *SCR&Tox*, DETECTIVE, NOTOX and COACH to participate in an interactive workshop to discuss bioreactors as well as cell engineering for liver engineering purposes. The investigators from the four consortia provided in-depth information regarding the type of cell engineering as well as bioreactors that are used within each consortium. This initial meeting enlightened the different consortia on the technologies used. It became clear that even if multiple consortia use bioreactors and engineer cells, the approaches are complementary. It was concluded that similar in-depth discussions at the yearly **SEURAT-1** meeting, and perhaps also in combination with some of the ***HeMiBio*** meetings, would be very important in allowing quick exchange of novel prototypes of biorecators and/or engineered cell populations between consortia. A separate report about this meeting is available in chapter 4.10.9, and a follow-up joint meeting on 'Bioreactors and Cell Engineering' is planned for September 2013.

During the second Annual Meeting of the **SEURAT-1** Research Initiative in Barcelona (6-8 March 2013), ***HeMiBio*** used the Market Place session (see chapter 4.9.2) to discuss four issues. The overall aims were to continue the discussion from the Gent meeting on cell engineering and bioreactor designs, as well as to obtain information from other consortia regarding the design of bioreactors and engineered cell populations. The following issues were discussed:

- Bioreactors and cell engineering (participants from NOTOX, DETECTIVE, *SCR&Tox*). Areas for discussion were (i) cells that are currently being made (engineering-wise); (ii) hepatocytes that are being used in the different consortia and appropriate references that could be used in the upcoming experiments (e.g., freshly isolated hepatocytes, freshly thawed hepatocytes, HepaRG cells); (iii) currently used bioreactors and readouts.

- Biomarkers (gene targets) for fibrosis, steatosis and cholestasis to be used for cell engineering (participants from ToxBank, DETECTIVE, *SCR&Tox*, Mode-of-Action Working Group). In this session, ***HeMiBio*** requested input regarding biomarkers to allow incorporation of molecular sensors in different cell components in the bioreactor.

- Range of toxins (drugs/cosmetics) and their metabolites expected *in vitro (*participants from ToxBank, DETECTIVE, COSMOS, NOTOX). In this session, ***HeMiBio*** requested input from ToxBank and other **SEURAT-1** partners related to levels of cosmetics and other toxic components that cause fibrosis, cholestasis and steatosis, and the metabolites of these molecules that can be measured in the cell and their surroundings, so that electrodes can be built for measuring these components in the dose range both in and out of the cell.

➠ Range of extracellular levels of cell metabolism molecules to be expected in bioreactors (participants from DETECTIVE, COSMOS, NOTOX). In this session, ***HeMiBio*** requested input on how to optimise electrodes for integration in the bioreactor. ***HeMiBio*** requested precise information regarding the extracellular levels that can be expected from a number of components (including ALT, LDH, glutamate, lactate and glucose) per amount of cells in an *in vitro* setup.

We obtained significant input from these Market Place session on how to proceed with development of the engineered cells and the creation of the bioreactor, in order to make them suitable for repeat toxicity testing.

As immediate practical points, we will: (i) collaborate with Christophe Chesne from *SCR&Tox* to assess the HepaRG cell line and determine if the engineering done in PSCs by ***HeMiBio*** can also be accomplished in HepaRG cells; (ii) compare the suitability of the Upcyte® hepatocytes, PSC-derived hepatocytes and HepaRG cells for repeat toxicity testing; (iii) collaborate with Bob van de Water (DETECTIVE) who has developed a large series of HePG2 cells with constructs that allow toxicity imaging, to see whether (parts of) the constructs used in DETECTIVE could also be used to engineer PSCs and their progeny; (iv) start testing the retention of drugs/cosmetics to be used in repeat toxicity testing in the bioreactor, in collaboration with the Biokinetics Working Group.

Preceding the second-year ***HeMiBio*** consortium meeting in Barcelona, Spain, on 16 January 2013, a winter school, open to non-***HeMiBio*** members, was organised by Mathieu Vinken and Vera Rogiers (Vrije Universiteit Brussels, Department of Toxicology, Belgium), entitled: Concepts of Toxicology and Risk Assessment. The contents of this winter school are summarised elsewhere (see chapter 4.11.1). As a follow up for the winter school, ***HeMiBio*** plans a summer school on 4–6 June 2013, open to non-***HeMiBio*** members, organised by Prof. Mathieu Vinken and Prof. Vera Rogiers (Vrije Universiteit Brussels, Department of Toxicology, Belgium), entitled: Practical Concepts of *in vitro* and *in silico* Toxicology (see short description in chapter 4.11.1).

HeMiBio has provided input into several **SEURAT-1** Working Groups and activities, including the selection of cross-cluster reference compounds for toxicity testing, the selection of modes-of-action to be addressed and the development of case studies for repeated dose toxicity. The most active ***HeMiBio*** partner in this context is the Vrije Universiteit Brussels group, who is also involved in the DETECTIVE project, which focuses on the identification of *in vivo*-relevant *in vitro* biomarkers for repeated dose systemic toxicity. Because of this unique position, the Vrije Universiteit Brussels group is able to contribute to the establishment of continuity, transparency and intensive collaboration between projects of the **SEURAT-1** Research Initiative, as was requested in the original EC-Cosmetics Europe project call. Specifically, this partner has generated as much as 15 standard operating procedures, describing methods related to functionality and drug-induced liver toxicity testing in cultured liver cells, which will

be used consistently by both consortia and, as this information was also fed into the ToxBank Data Warehouse, potentially other **SEURAT-1** projects. The same holds true for the lists of chemicals to be tested, which have been compiled by the Vrije Universiteit Brussels group based upon extensive discussions with the DETECTIVE and ***HeMiBio*** partners. In addition, the Vrije Universiteit Brussels group also foresees continuous interaction with the ToxBank project, by acting as a spokesperson for both DETECTIVE and ***HeMiBio*** during ToxBank meetings and by sharing the DETECTIVE and ***HeMiBio*** standard operating procedures and information regarding the selection of compounds. Furthermore, Mathieu Vinken from the Vrije Universiteit Brussels group is a member of the **SEURAT-1** Safety Assessment Working Group and recently became co-leader of the **SEURAT-1** Mode-of-Action Working Group, both of which are working towards the cluster-level objectives of the **SEURAT-1** Research Initiative. The following **SEURAT-1** workshops have been attended on behalf of ***HeMiBio***:

- Describing mode-of-action in liver toxicity using adverse outcome pathways, 24–25 October 2012 in Ispra, Italy.
- The development of case studies to define fit-for-purpose risk assessment of repeated dose systemic toxicity, 13–14 November 2012 in Tübingen, Germany.
- Building shared experience to advance practical application of pathway-based toxicity: liver toxicity mode-of-action, 23–25 January in Baltimore, USA.
- ***HeMiBio***-ReLiver workshop, 20–22 February 2013 in Heidelberg, Germany.

4.3.7 Expected Progress within the Third Year

Finalisation of the molecular and epigenetic characterisation of primary liver-derived zonated hepatocytes, LSECs and HSCs: Little data is available on the expression profiling or methylation profiling of human uncultured HSCs, LSECs and zone-specific hepatocytes. In the third year we will further analyse the transcriptomic and epigenetic characterics of freshly isolated liver cell populations with cultured cells. Our transcriptomic analysis of qHSCs versus culture-activated HSCs confirmed many 'pro-fibrotic' markers that we found associated with the activated phenotype of human HSCs, but also identified several new ones. We will investigate whether these markers can be used as *in vitro* readouts of HSC activation (a hallmark of fibrosis) in mono-cultures and co-cultures of HSCs. From this transcriptomic analysis we also identified a set of 90 genes that could act as upstream transcriptional regulators of a range of pro-fibrogenic genes that were found regulated in our gene arrays. The generated list contains genes known to play a pivotal role in fibrosis, such as connective tissue growth factor (CTGF), but also genes that have never been described in the context of fibrosis and/or HSC biology. Our research will focus on regulators, the activity of which could be measured *in vitro* using reporter assays, and these could eventually be built in hiPSCs. A first selection of

interesting upstream regulators will be made based on the gene function and fold difference in expression between qHSCs and culture aHSCs. This initial selection will further be refined by testing the genes for their relevance in human disease. This will be done by localising and measuring their levels of expression in total liver fractions of healthy and cirrhotic patients as well as immunohistochemical analysis of healthy and diseased liver sections.

Our methylation profiling of the freshly isolated liver cell types yielded a set of methylated genes specific for HSC activation, meaning genes up-regulated by activation (>5 fold during culture activation) which are hyper methylated in qHSCs. Likewise, we identified down-regulated genes (>5 fold during culture activation), which are hyper methylated in aHSCs. We will analyse and quantify the differential methylation state between qHSCs and culture aHSCs using bisulfite sequencing, and try to confirm this for *in vivo*-activated HSCs by isolating HSCs from fibrotic/cirrhotic tissue samples (currently being optimised). The described methylation profiling will also provide for comparison of future hiPSC-derived HSC-like cells with human primary HSCs.

Further integration of the miRNA profiles into the gene regulatory pathways and methylation status of genes involved in HSC activation will be necessary to get a complete view of HSC activation *in vitro* at the transcriptome level. The possible identification of specific miRNAs that regulate entire gene networks known to play important roles during activation of HSCs could be used to optimise our hiPSCs towards a HSC differentiation strategy or serve as an indication of activation status in *in vitro* cultures.

To create organoids that represent the liver, we need to take into account that hepatocytes (and non-parenchymal cells) are zonated. Although this has been assessed in murine livers, little data is available from human livers. Using laser capture microscopy we have isolated periportal and perivenous cells; the transcriptome as well as miRNAs expressed in the two different zones are currently being examined. These analyses will be completed in the next three to four months. Information gained from these studies will then be used for selection of different subpopulations of hepatocytes from PSC progeny.

PSC lines with the molecular sensors needed to assess fibrosis: Within **SEURAT-1**, ***HeMiBio*** is best placed to study fibrosis, as we combine multiple liver cells in a single bioreactor. In order to monitor the fibrogenic response of HSCs *in vitro*, some markers able to detect early or late events have been considered. Plasmid constructs incorporating established HSC activation markers as well as more general cell-stress response promoters will be established and tested in primary HSCs. If adequately responsive, these reporter constructs will be recombined in the AAVS1 locus of engineered iPSCs. Once hiPSC-derived HSC-like cells have been established, these genes with their reporters will serve as markers for HSC activation in response to *in vitro* stimulation with a fibrogenic drug in co-culture experiments and ultimately in a bioreactor setup.

We will further elaborate on Zinc finger nuclease-mediated homologous recombination and insert additional marker cassettes in the different cell types (hepatocytes, stellate cells and endothelial cells) in the third year of ***HeMiBio***. Cell culture conditions will be optimised for the expansion of quiescent iPSC-derived cells. Ion-selective micorelectrodes will be optimised and equipped with a preconcentration stage, and prototype 3D-bioreactors will be made available by the end of the third year.

ZFN- or TALEn-mediated homologous recombination in Upcyte® hepatocytes and LSECs: So far it has not been possible to complete homologous recombination in hepatocytes treated with Upcyte® protocols. An intense collaboration between ***HeMiBio*** partner Medicyte and the groups at Freiburg University and KU Leuven will further determine if this can be accomplished.

3D flow-through bioreactor, including the switchboard and the final layout: The complete system will consist of different modules: the bioreactor, the fluidic switchboard and the sensor module (including read-out). The different modules will be connected by standard HPLC fluidic connectors. The bioreactor can be either a 2D flow-over reactor, or a 3D flow-through reactor. Assembly of the 3D flow-through microfluidic bioreactor is a technological challenge. Therefore two paths will be followed: (i) realisation of the flow-through reactor in soft PDMS (silicone rubber) materials, allowing relatively easy assembly and short cycles of subsequent design optimisation; (ii) development of rigid polymer- (e.g., COC) based assembly technology and finally realisation of the flow-through reactor in this material, using the optimised design from (i). This technology is more challenging but has higher potential for industrialisation and up-scaling is expected.

4.3.8 Future Perspectives

HeMiBio is currently focused on generating a bioreactor that mimics the architecture and different cellular components present in liver sinusoids. The technology developed for this bioreactor (i.e., microfluidics and spatial isolation technologies; the development of sensor modules directed towards medium composition, e.g., pH, oxygen, glucose etc., as well as cell toxicity detection; and the master stem cell lines allowing easy introduction of lineage-specific promoter constructs or toxicity detector gene sequences), should be transferrable to other bioreactors.

For instance, the endocrine cells of the pancreas exist as clusters called islets of Langerhans. The insulin-producing beta cells are part of these islets, and when damaged, type I or type II diabetes ensues. Microfluidic devices for high-throughput and online monitoring of insulin

secretion from individual mouse pancreatic islets in parallel have been developed, allowing testing of lipotoxicity by free fatty acids. Hence, *in vitro* monitoring of insulin production combined with changes/toxicity to specific cells within islets as described in ***HeMiBio*** for the liver can be used for toxicity testing in general or rapid evaluation of islets for transplantation (*Dishinger et al., 2009*). To replace the beta cells, it is now possible to graft islets, but effective strategies to develop islet transplantation for widespread clinical application will require effective measures against current problems such as vascularisation, immune-mediated rejection and shortage of tissue to transplant. Expansion of islet-like tissue in bioreactors has been achieved starting with neonatal porcine pancreatic cells (*Chawla et al., 2006*). As an alternative source, islet-like clusters able to synthesise and secrete insulin can be derived from hES cells and hiPS cells, and pancreatic endoderm derived from hES cells efficiently generated glucose-responsive endocrine cells after implantation into mice (*Madsen, 2005; D'Amour et al., 2006; Zaret & Grompe 2008*). Thus, selection of immature cells derived from hiPS cells and further differentiation in suitable 2D-/3D-bioreactors, which will be developed in ***HeMiBio***, could serve to improve beta cell differentiation and the development of more complex pancreatic bioreactors.

The technologies developed in ***HeMiBio*** could also be used to create a kidney-simulating device. The human kidney, like the liver, is important for detoxification of the blood. Although dialysis can be used to detoxify the blood of patients with renal failure, they suffer from significant remaining toxicity and early mortality. The kidney can is composed of approximately 1.2 million individual nephrons working in parallel. Each nephron can be divided into 3 main components. Blood flows into the nephron, first entering the glomerulus, where the blood is filtered by passive mechanical filtration through fenestrated endothelium, retaining cells and large proteins. From there, blood and filtrate flow to the proximal tubule, where large amounts of solute and fluid are actively reabsorbed. Finally, the blood and filtrate flow to the loop of Henle and associated collecting ducts. In this part of the nephron, active pumping, osmosis and diffusion combine to reabsorb almost all of the remaining filtrate fluid, resulting in highly concentrated waste urine. Several methods have been developed to isolate glomeruli and culture the three types of glomerular cells. For instance, the concept of a nephron-on-a-chip using a MEMS-based (MicroElectroMechanical System) bioartificial device has been proposed, but attempts to populate this device with the various renal cell types that constitute a kidney have not been reported (*Weinberg et al., 2008*). However, the methods suffer from impure cell populations and the short lifespan of the cells cultured *in vitro*. *In vitro* reconstruction of the glomerulus using co-culture in combination with collagen vitrigel has been partly successful; glomerular epithelial cells (podocytes) and mesangial cells maintained cell growth and cell viability for up to one month, forming a 3D-dimensional glomerular organoid (*Wang & Takezawa, 2005*). The population of 2D- and 3D-bioreactors with hiPS cell-derived cultures, enabling life imaging and monitoring of the differentiated cell types as is presented by ***HeMiBio***, could also be used to develop bioartificial renal technology.

Although the liver is the principal organ to clear toxins from the body and therefore is the most vulnerable target for the latter, certain drugs may be toxic to other vital organs, such as the heart, the blood vessels or the brain. In order to predict toxicity of cosmetic compounds or drugs to these organ tissues, creation of devices that mimic their architecture and function for toxicity screening is of great importance. As with the liver, the functional, morphological and molecular characteristics of the cells that constitute these organs are determined by environmental factors (e.g., the vicinity to and direct contact with other cell types in the organ, the exposure to flow and certain oxygen levels, etc.). All these parameters can be integrated in a bioreactor system, such as the one we propose here for the liver. The technologies developed in ***HeMiBio***, that is, (i) cells that are manipulated so that their differentiation state, functionality and viability can be monitored, and (ii) the inclusion of sensors that can monitor the environment of the cells, can be translated to other organ systems for high-throughput screening for the effect of drug candidates without needing animals.

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4.4 DETECTIVE: Detection of Endpoints and Biomarkers for Repeated Dose Toxicity using *in vitro* Systems

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4.4.1 Introduction and Objectives

The assessment of repeated dose toxicity is a standard requirement in human safety evaluation and relies on animal testing, as no alternatives are currently accepted for regulatory purposes. In the first step towards replacement of *in vivo* repeated dose systemic toxicity testing, the **DETECTIVE** project, therefore, focuses on the identification of robust, reliable, sensitive and specific biomarkers indicative for repeated dose toxicity of specific compounds in the *in vitro* systems.

During the investigation of hepatotoxic, cardiotoxic and nephrotoxic effects of selected compounds, it is expected that **DETECTIVE** will be able to define human toxicity pathways relevant for these organs (liver, heart and kidney). Upon systematic exploitation of complementary functional and '-omics' readouts, the project aims to identify and develop human biomarkers in these cellular models suitable for repeated dose *in vitro* testing. As functional readouts investigate the effects of toxicants on specific cell functions, a battery of complementary '-omics' techniques will deliver comprehensive data on the cellular situation at the molecular level.

To achieve the identification of biomarkers for repeated dose systemic toxicity, the specific objectives of **DETECTIVE,** which were addressed in the second year, are:

- to interface with the other building blocks of the **SEURAT-1** Research Initiative, in particular ToxBank, to substantiate the knowledge of toxicological data on relevant compounds, as well as existing biomarkers for chronic organ damage such as cardiomyopathies, arrhythmias, liver cirrhosis, steatosis, cholestasis, apoptosis, etc.;

- in collaboration with *SCR&Tox*, to evaluate the suitability and robustness of existing cell lines for use in developing biomarkers for repeated dose toxicity testing *in vitro*;
- to analyse the basic questions related to chronic toxicity testing, including studies on the duration of compound exposure that causes toxicity, factors assisting in reversibility of effects, long-term impact of modified genes, etc.;
- to develop functional readouts in human *in vitro* model systems mainly for liver, heart and kidney. These functional parameters include (i) electrical activity (ECG-like, MEA); (ii) impedance measurements; (iii) imaging; and (iv) cell-specific functional readouts such as enzyme activities, cytokine release, albumin and urea secretion, glycogen uptake, cholestasis, steatosis, and protein release from target cells;
- to develop '-omics' readouts in human *in vitro* model systems for liver, heart and kidney. These '-omics' readouts include: (i) integrative transcriptomics (microarrays for global screening of gene expression, epigenetics, and miRNA); (ii) proteomics; and (iii) metabonomics.
- to develop concepts for a standardised approach employing appropriate cellular model systems that: (i) determine the best biomarker candidates for toxicity assessments through identification of associated adverse outcome pathways, focusing also on reproducibility (biomarker qualification); and (ii) distinguish sensitive and target-specific biomarkers from generic cellular stress effects.
- to systematically organise data with the use of standardised nomenclature that facilitates the online sharing of biomarker metadata.

In this report, we present several highlights from experiments on hepatotoxicity, cardiotoxicity and nephrotoxicity in the second project year (sections 4.4.3–4.4.6). In particular, the report describes:

- the establishment of cultivation and analytical conditions to assess molecular mechanisms of toxicity in primary human hepatocytes (PHH);
- the quantification of biomarkers of cytotoxicity in HepG2 BAC-GFP cell lines using high-content imaging;
- the establishment of a repeated dose toxicity protocol in commercial human iPS-derived cardiomyocytes;
- functional and '-omics' experiments in kidney cells exposed to different compounds.

4.4.2 Main Achievements and Challenges in the Second Year

The major achievements of the second year were initiated with the successful design of the exposure protocol for repeated dose toxicity testing suitable for the different organ groups. Furthermore, each target organ group – liver, heart and kidney – focused on developing a complete toxicological profile with the selected reference compounds based on both functional and '-omics' analyses.

Development of the different functional readouts and adaptation of these technologies to long-term toxicity testing have been continued during the second project year. The complete workflow for sample preparation, labelling, shipping, application in subsequent epigenetic analyses and integrated data analyses of epigenetic and microRNA data, has been successfully developed.

The forthcoming deliverables and milestones will focus on the individual high-throughput functional and '-omics' readouts and the identification and validation of the final list of biomarkers.

Biomarkers for Hepatotoxicity

In the first year, **DETECTIVE** chose PHH as the gold standard cell system for the identification of biomarkers of hepatotoxicity. However, as other cell lines are more suitable for some particular readouts, work has been carried out using also HepG2-, HepaRG- and hSKP-HPC-based cell systems.

PHH cultivation conditions for long-term toxicity testing and '-omics' analysis have been successfully established and carried out. Imaging analysis of the PHH after acetaminophen exposure has clearly indicated early, middle and late apoptotic features. The liver group has also successfully established cultivation and analytical conditions to assess molecular mechanisms of toxicity in PHH (see section 4.4.3). Additionally, the proteomic quantitation methods on PHH were successfully optimised. For this, a preliminary protein recovery from primary hepatocytes (rat model) using the strong anion exchange (SAX) fractionation technique (*Wiśniewski et al., 2009*) was conducted, and recorded a marked improvement of 35% in the protein recovery from samples. Furthermore, first repeated dose toxicity experiments using compound-treated versus time-control human hepatocyte samples were conducted at different time points. Relative quantitation was done using iTRAQ-technology (*Ross et al., 2004*) and high-speed and high-resolution mass spectrometers.

In a different study, a successful BAC-GFP transgenomic approach has helped to establish a set of HepG2 BAC reporter cell lines that define perturbations in the major adaptive response pathways (oxidative stress, DNA damage and ER stress) and also defined mitochondrial morphology and cytochrome-c release. A high-content imaging approach has been successfully

established with dynamic cellular stress responses being quantified by a specially designed automated image analysis pipeline (see section 4.4.4). These reporter cell lines have been tested for their functionality using model compounds and various drug-induced liver injury (DILI) compounds which specifically activate the anticipated reporters. Lastly, the application of the established reporter systems in RNAi approaches and compound screens has also been confirmed. The meta-analysis strategy of the TG-GATES dataset (Japanese Toxicogenomics Project) was applied in the context of compound-induced liver injury: gene expression datasets of PHH helped to identify new candidate biomarkers for future BAC reporter cell lines.

Thirteen liver toxicants were tested in a HepaRG cell model using high-content multicolour cell imaging and selected staining probes measuring the mitochondrial dysfunction. The results indicate the potential for using these techniques in the assessment of adverse effects in the mitochondrial compartment.

hSKP-HPC cells exposed to acetaminophen showed the same hepatotoxic expression patterns as PHH cultures treated with the same compound. Moreover, there were a higher number of genes commonly modulated in hSKP-HPC cells than in PHH cultures exposed to acetaminophen, suggesting a higher predictive capacity of hSKP-HPC cells. A list of genes with modulated expression has been identified. This gene set will be further investigated with the intent of defining possible biomarkers for hepatotoxicity.

Biomarkers for Cardiotoxicity

Cardiomyocytes were analysed for arrhythmias, disturbance of cardiac conductance, Ca^{2+} pathways and inhibition or impairment of cardiac contractility. The electrophysiological characteristics of cardiomyocytes were monitored in real time using MEA and patch clamp technologies. While obtaining a monolayer of the cardiomyocytes for MEA measurement was quite challenging, the patch clamp technology has been successfully applied to defining cardiomyocyte response to acute exposure of selected standard reference compounds. Cardiomyocyte response to repeated dose exposure of one reference compound (mannitol served as a negative control) has been assessed using impedance technology. Results showed arrhythmic behaviour in cardiomyocytes at the defined concentration while higher doses proved cytotoxic. A washout study showed that the cardiomyocyte responses to repeated doses of the reference compound could not be improved following the drug removal. The impedance technology has also been used for dose response determination after long-term treatment of the cardiomyocytes with the two other reference compounds.

Mitochondrial dysfunction was found to be a key event in reference compound-induced cardiotoxicity. Both transcriptomic and metabolomic data have supported this observation. High-content imaging techniques were developed assessing mitochondrial morphology, mitochondrial membrane potential, reactive oxygen species level and ATP content. This

represented an adaptation of the original work plan for investigating the chronotropic effects in response to the reference compound exposure.

Even though whole-genome profiling of cardiomyocytes has shown that they can be used for toxicity studies for at least 14 days (see section 4.4.5), only four treatments of the reference compound at the chosen concentration were possible in the repeated dose toxicity model showing cytotoxicity at higher doses. Transcriptomic data has shown that the reference compound treatment caused down-regulation of several genes involved in heart contraction at early time points and in a time- and dose-dependent manner. Metabolomic analysis of cell culture media has shown that NMR-based metabolic profiling can detect the reference compound-associated perturbations in mitochondrial metabolism at early time points and in a time- and dose-dependent manner (e.g., pyruvate and 3-hydroxyisobutyrate concentration).

Biomarkers for Nephrotoxicity

Several functional readouts were employed, assessing the effects of compounds on repeated exposure of renal epithelial cells (RPTEC/TERT1). These readouts included impedance, glycolysis rate, quantification of reduced glutathione (GSH) and cellular ATP. The impedance measurement system in particular has been further optimised for long-term observation of the epithelial monolayer integrity in the renal cell line. Six nephrotoxins in total (chosen based on advice received from ToxBank) were tested, and two of them were chosen for comprehensive '-omics' analysis. The experimental outcome helped in defining the first set of biomarkers for renal toxicity. Furthermore, experimental protocols have been optimised for successful isolation of microRNAs and RNAs as well as simultaneous extraction of metabolites and proteins from the same samples. The '-omics' analysis has been conducted and the data analysis is under progress. The preliminary results from the transcriptomic analysis of the kidney samples have shown a deep biological impact of the compounds with clear time effects (see section 4.4.6).

The DETECTIVE Database

The infrastructure for collecting, storing and sharing the experimental data of the **DETECTIVE** consortium has been successfully set up based on standard formats such as ISATAB. It will deliver a solution that is tailored specifically to the **DETECTIVE** needs. Preliminary experiments were used for developing the working database in order to offer a user-friendly input system, an annotation convention and a sample labelling standard. From now on, the main focus of work will be data collection and integration of solutions provided by ToxBank into the **DETECTIVE** database.

The future acceptance and use of biomarkers for regulatory purposes is a major task that

has yet to be accomplished. Indeed, it requires a set of quality evaluations to determine the scientific validity of the proposed biomarkers, such as information on the predictivity of the biomarker itself, but also the methodologies by which it can be assessed.

4.4.3 Cultivation and Analytical Conditions to Assess Molecular Mechanisms of Toxicity in Primary Human Hepatocytes

Introduction

Studies on mechanisms of hepatotoxicity have been largely aimed at biochemical activation of drugs and chemicals to reactive intermediates, which are considered to be the direct cause of cell damage. However, in recent years it has become clear that this is only partially true. For example, hepatotoxicity induced by acetaminophen has been shown to be strictly dependent on the activation of stress-signalling pathways such as JNK (*Kaplowitz et al., 2008; Gunawan et al., 2006*). The activation of stress pathways may occur at intracellular organelles such as the endoplasmic reticulum (ER). Upon accumulation of misfolded proteins, specific sensors located at the ER (e.g., IRE1α, PERK and ATF6) become activated and trigger a constellation of signalling pathways, which can lead to cell death (*Malhi et al., 2011*). Importantly, the ER-stress sensor IRE1α has been shown to be a major hub for integration of signalling adaptors such as TRAF2 and ASK1, which mediate activation of JNK (*Hetz et al., 2011*). Therefore, it is of utmost importance that novel *in vitro* systems aimed at replacing animals for toxicity testing are able to reproduce all aspects of drug-/chemical-induced toxicity, including metabolic activation and further signalling pathways relevant for these effects.

At a cellular level, primary hepatocytes represent a gold standard for *in vitro* systems aimed at detection of hepatotoxic effects (*Hewitt et al., 2007*). However, these cells undergo dedifferentiation during cultivation, which results in progressive loss of function and features of epithelial to mesenchymal transition (*Zellmer et al., 2010*). This can be partially prevented by using an improved 3D extracellular matrix, such as collagen gel in a 'sandwich' configuration (*Beken et al., 1997; 2001; De Smet et al., 2001a; 2001b; Godoy et al., 2009; 2010a; 2010b*). Nevertheless, it is not known to what extent primary hepatocytes can reproduce all *in vivo* aspects of drug-/chemical-induced toxicity, including stress-signal transduction events. In **DETECTIVE**, one aim is therefore to establish *in vitro* systems using PHH that fully reproduce the most important aspects of drug-/chemical-induced hepatotoxicity, including metabolic activation of compounds and further downstream stress-signalling pathways relevant for toxicity.

Approach

To identify molecular mechanisms of hepatotoxicity at epigenomic (e.g. DNA methylation), mRNA and protein levels in primary hepatocytes, we have applied our extensive knowledge

of *in vitro* systems and generated cultures of PHH in collagen sandwich using state-of-the-art procedures for toxicity testing. In brief, freshly isolated hepatocytes from three independent human donors were plated between two layers of collagen gel (collagen 'sandwich'), and exposed to mM acetaminophen for 30 minutes, 2, 4 and 24 hours, and 7 days. We optimised procedures for isolation of mRNA, DNA and proteins for gene array analysis and proteomics, respectively. Control samples were collected at each time point. Additionally, we analysed by western blot the expression of IRE1α in freshly isolated hepatocytes and at different time points during cultivation. The presence of a 3D extracellular matrix represented an important challenge for proteomic analysis; therefore, we conducted preliminary assays using mouse hepatocytes to ensure optimal protein extraction for robust and sensitive analysis in human hepatocyte samples.

Results

The PHH are cultured in a 3D extracellular matrix, which presented potential complications in isolation of cell extracts intended for the '-omics' analysis. Through close collaboration with the University of Saarland and partners from the NOTOX project, a procedure was established to isolate RNA and DNA from the same sample for the transcriptomic and epigenomic readouts. For proteomics, the protein extraction protocol is optimised for Rapigest®-based protein extraction, which is the method of choice for proteomic analysis.

After establishing a stable cultivation protocol, long-term repeated exposure experiments using acetaminophen (APAP) were carried out. Preliminary analysis revealed a strong response in both RNA and protein levels, with several hundred genes/proteins being altered by APAP toxicity (not shown). Imaging analysis of the PHH after APAP exposure has clearly indicated early, middle and late apoptotic features (*Figure 4.24*). **DETECTIVE** has thus successfully established cultivation and analytical conditions to assess molecular mechanisms of toxicity in PHH.

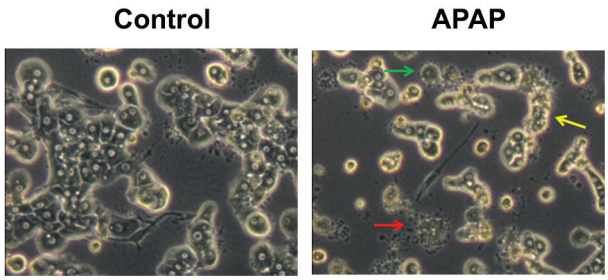

Figure 4.24 *Phase contrast images of PHH after 7 days in culture in control and 1 mM APAP. Early, middle and late apoptotic features (green, yellow and red arrows respectively) are clearly observed after APAP exposure. Data source: Leibniz Research Centre for Working Environment and Human Factors, Germany.*

The importance of signal transduction pathways as significant readouts in toxicity testing was recently demonstrated using the gene JNK (*Gunawan et al., 2006*). Earlier studies on *in vitro* and *in vivo* murine liver models confirm that stress-signal transduction pathways are triggered by APAP toxicity *in vivo*, and also support a potential role for ER-stress responses in cell death. Since ER-stress pathways represent an important mechanism for hepatotoxicity, it is important that *in vitro* systems preserve the machinery necessary to engage these responses, in order to fully reproduce all signalling and metabolic events of toxicity.

In **DETECTIVE**, we assessed the expression level of important ER-stress sensor IRE1a (*Figure 4.25*) in cultivated hepatocytes and compared it to that of liver tissue. The results indicate that current cultivation techniques of primary hepatocytes must still be improved to fully reflect *in vivo* properties.

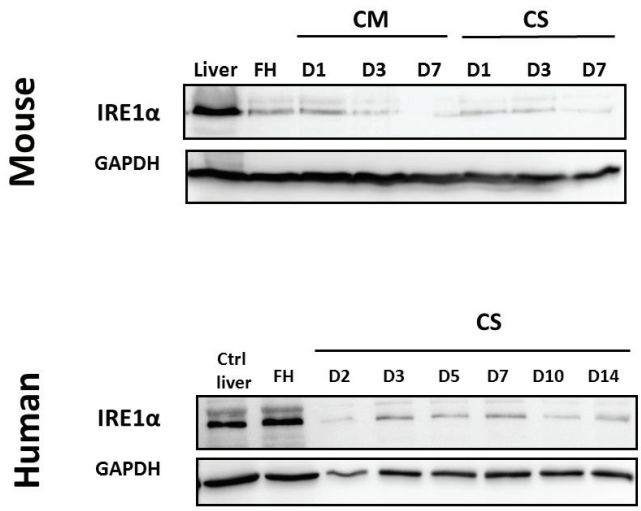

Figure 4.25 *Downregulation of IRE1a in cultivated primary hepatocytes. Total protein extracts were generated from either mouse or human primary hepatocytes and from mouse or human liver tissue. The extracts were blotted and probed with an antibody against IRE1a. In both cases, a clear downregulation of IRE1a is observed in cultivated hepatocytes compared to liver tissue. Data source: Leibniz Research Centre for Working Environment and Human Factors, Germany.*

As there is very little information available on the mechanisms regulating expression of ER-stress sensors, we will focus in the coming year on identifying the mechanisms leading to alterations in ER-stress sensors and, more particularly, on the mechanism behind severe downregulation of IRE1a in cultivated hepatocytes.

4.4.4 Characterisation of BAC-GFP HepG2 Reporter Cell Lines using High-Content Imaging

Introduction

The eventual outcome of (compound-) induced toxicity at the tissue or whole body level is usually dependent on damage at the molecular level. Molecular level damage can lead to perturbations on the cellular level, e.g., damaged organelles, damaged cytoskeletal structures, oxidative stress, DNA damage and the resulting adaptive stress-response pathways. The toxicity origin and corresponding early cellular response is often referred to as 'mode-of-action' (MoA).

Many academic and industry groups have set up cellular-based toxicity screening systems to try to capture cellular level compound-induced toxicity. However, many of these systems are based on end-point assays, e.g., cell death by apoptosis/necrosis and mitochondrial membrane potential. Others are based on standard reporter transcription-based assay systems, e.g., luciferase-based reporters that will not allow a dynamic cell-to-cell based analysis. The application of BAC-GFP reporter cell lines ensures that the observed reporter responses are based on *endogenously regulated genes* and, in addition, that the readouts are based on the *transcriptional and translational responses*. Using a panel of BAC-GFP reporter cell lines and automated high-content confocal imaging, we can observe the time-resolved dynamic cellular stress responses at the organelle level, as well as all major cellular (mal) adaptive stress-response pathways, for various concentrations. We have shown that the reporter cell lines respond to their corresponding model stress-inducing compounds. We anticipate that the combined information of the response of a panel of reporters to compounds will improve toxicity prediction and direct us to the compound-specific mode-of-action.

Approach

A set of HepG2 BAC reporter cell lines that define perturbations in the major adaptive response pathways (oxidative stress, DNA-damage, mitochondrial organisation and ER stress/UPR (unfolded protein response pathway)) has successfully been established. Target markers are identified through a data mining approach and developed into BAC-GFPs using BAC transgenomics. These BAC-GFPs are then transfected into mammalian HepG2 cells. Previously published results using mouse embryonic stem cells show that the Srxn1 gene is an excellent reporter for oxidative stress (*Hendriks et al., 2012*). For the current study, novel biomarker genes (including Srxn1, KEAP1, Nrf2, DDIT3/Chop, ATF4, CYC1 and CYCS) that are modulated by certain groups of DILI (drug-induced liver injury) drugs were identified through mining the Japanese Toxicogenomics (TG-GATES) dataset. The compounds were classified according to FDA-approved drug-labelling for DILI-severity (*Tong et al., 2011*). Based on previous gene expression profiling studies, we defined the genes that represent

the most important adaptive stress-response pathways in PHH. Through RNAi experiments, pathways relevant to HepG2 cytotoxicity were identified and found to include oxidative stress, the unfolded protein response and apoptosis pathways. The data support the overlap in stress-response pathways in PHH with HepG2 cells and indicate that reporter systems in HepG2 cells will be reminiscent for the studied stress pathways in PHH. Bioactivation-dependent toxicity testing will be further investigated by culturing the HepG2 reporter lines in a more physiologically relevant 3D environment, such as spheroids. Alternatively, the use of BAC-GFP constructs to transform pluripotent stem cells lines that can be differentiated into hepatocytes will be investigated.

Results

A meta-analysis strategy of the TG-GATES drug-induced liver injury (DILI) gene expression dataset in PHH was followed to identify new candidate biomarkers as candidate genes for development of our BAC reporter cell lines. The meta-analysis has helped to identify the expression of DDIT3/CHOP as a critical marker for cell injury for different DILI compounds. Besides, the data support overlap in stress-response pathways in PHH compared to HepG2 cells and indicate that reporter systems in HepG2 cells will be reminiscent for the stress pathways in PHH. These reporter cell lines have been tested for their functionality using model compounds and various DILI compounds, which specifically activate the anticipated reporters. Three compounds specific to each individual reporter panel were chosen.

A high-content imaging approach using confocal microscopy in a 384-well format has been successfully established for quantification of the resulting dynamic cellular stress responses, using a specially designed automated image analysis pipeline. This approach has helped define BAC-GFP reporters for oxidative stress, mitochondrial morphology and cytochrome-c release. Having identified the Srxn1 gene as an excellent reporter for oxidative stress in mouse embryonic stem cells (*Hendriks et al., 2012*), BAC-GFP reporters for Srxn1, KEAP1 and Nrf2 were generated for the analysis.

Using the high-content imaging approach, oxidative stress-inducing compounds, including iodoacetamide, diethyl maleate and menadione, were found to induce the appearance of KEAP1-GFP cytoplasmic foci. Further results demonstrated co-localisation of KEAP1-GFP with p62, indicative of autophagosome localisation of KEAP1 during oxidative stress.

Evaluation of BAC-GFP Nrf2 HepG2 cell lines after exposure to iodoacetamide shows a transient increase in the cellular levels of Nrf2-GFP with a primary location in the nuclear compartment. GFP-Srxn1 levels were monitored under control conditions and after iodoacetamide treatment. Results show induction of GFP-Srxn1 levels after treatment with iodoacetamide (*Figure 4.26*). In addition, the time course of the responses was found to follow

the KEAP1 stabilisation and localisation in foci. Similar responses were also observed for menadione and diethylmaleate exposures.

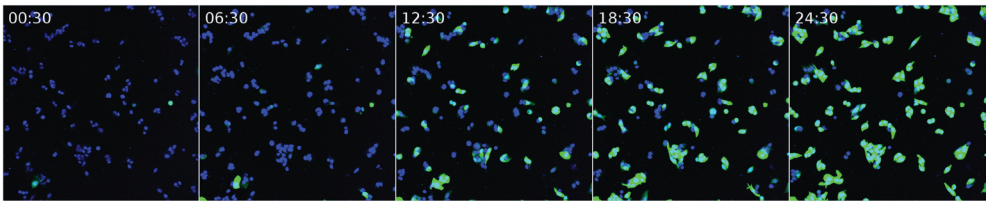

Figure 4.26 *Srxn1-GFP expression is induced after treatment with iodoacetamide. Data source: Universiteit Leiden, The Netherlands.*

In order to determine the feasibility for defining a concentration relationship we used the compound CDDO-Me, which has specificity to activate KEAP1 and thereby activate the Nrf2 response. We used an entire concentration range and could demonstrate a clear concentration response for the expression of Srxn1-GFP at a 24-hour time point.

Lastly, the effects of a mitochondrial toxicant oligomycin A on the mitochondria were studied using CYC1-GFP, which is solely located in the mitochondria. The results were then quantified using Custom ImageJ Algorithms. Similarly, cytochrome-c release from the mitochondria in response to cisplatin exposure before the onset of apoptosis could be analysed using CYCS-GFP cells.

4.4.5 Repeated Dose Toxicity Protocol in Human iPSC-derived Cardiomyocytes

Introduction

In the context of cosmetics, which are generally used for months and years, assessment of long-term repeated dose toxicity is of particular importance. Currently, repeated dose organ toxicity is assessed in animal models, e.g., after 28 and 90 days of exposure in rodents or non-rodents (depending on route of exposure: OECD TG407-413), and is predominantly derived from histopathology and clinical chemistry. Only a few attempts have been made to obtain toxicological data from long-term exposure of cell or tissue culture systems to toxic substances or pharmaceuticals (*Pfaller et al., 2001*). In order to assess the long-term effects of repeated dose toxicity testing, it was first necessary to develop an exposure protocol in an *in vitro* human cardiac model.

An *in vitro* repeated dose toxicity study provides a certain level of flexibility and variability in the designing of an exposure protocol, e.g., with respect to choosing the appropriate cellular model,

duration of the study and time points for each exposure. Human iPSC-derived cardiomyocytes with typical electrophysiological characteristics (*Jonsson et al., 2011*) have been used in our research to design an *in vitro* predictive cardiotoxicity testing study integrated with a battery of high-throughput functional and '-omics' technologies for detecting early toxicity markers.

Approach

Establishing an exposure protocol for repeated dose toxicity testing in an *in vitro* cardiomyocyte model is quite challenging. With the growing interest in *in vitro* toxicity testing, our goal was to develop a standard protocol for repeated dose toxicity testing in human ES- or iPS-derived cardiomyocytes. Consequently, studies were conducted using human iPSC-derived cardiomyocytes exposed to a series of test concentrations of a reference compound. The results recorded a dose-dependent cytotoxicity and arrhythmicity in the cardiomyocytes after a 48-hour exposure to the test substance. The cytotoxicity study helped to develop a dose-response curve, from which we could identify two sub-lethal concentrations for the repeated dose toxicity testing in our *in vitro* cardiomyocyte model.

According to the long-term aim of establishing an adverse outcome pathway explaining the adverse effect of the test substance on the heart, the definition of an optimal exposure protocol was derived. Since the mechanistic basis of chronic effects on the heart as observed in patients is not clearly defined, we conducted a guiding experiment at transcriptome level in order to:

- dentify the most suitable readouts for a higher functional level;
- define optimal exposure times related to the selected readouts;
- monitor 'adaptive' stress responses at genome level;
- identify candidate biomarkers;
- determine the 'point of no return' at which impaired functionality cannot be compensated for at cellular level (note that it is beyond the remit of **DETECTIVE** to determine whether the malfunction of the cardiomyocyte can be compensated for at organ (system) level);
- assess the reversibility of the observed effects.

Repeated dose toxicity testing using the selected sub-lethal doses of the compound for cytotoxicity has revealed that both the doses, even though defined as sub-lethal by earlier studies, on repeated exposures could cause cytotoxicity as early as day six. Hence, based on these results, the exposure protocol has been revised so that the current study involves testing both toxicity and recovery in the *in vitro* model.

Results

Two sub-lethal doses of a reference compound were chosen based on results from earlier studies. The cardiomyocytes were then exposed to the test substance for 3 hours, 2 days, 4 days, 6 days, 8 days, 10 days, 12 days and 14 days as shown in *Figure 4.27*. The exposure protocol was designed so that the recovery of the cells after the drug exposure could also be studied; the cells were exposed to the test substance after every 48 hours and the sample collection was carried out as illustrated.

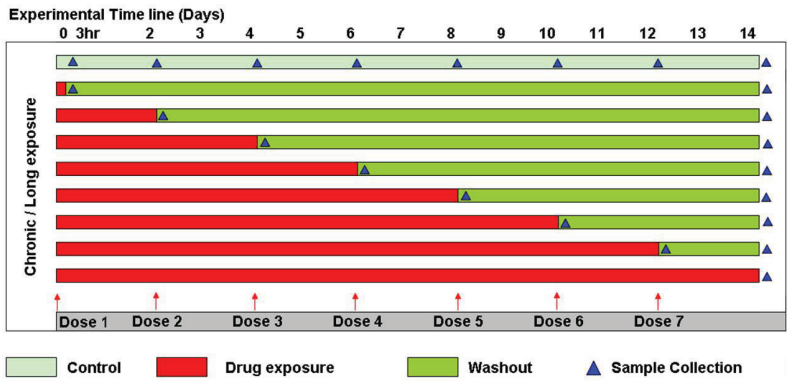

Figure 4.27 *The experimental design and timeline for repeated dose toxicity studies in human iPSC-derived cardiomyocytes. Data source: University of Cologne, Germany.*

The results indicate that the cardiomyocytes exhibit a dose-dependent increase in beating frequency as compared to the control cells. Moreover, the cells from day 2-, day 4- and day 6-treated batches could not show recovery even after washout. Results also indicate that the two sublethal concentrations identified could also cause cytotoxicity in the cardiomyocytes on repeated exposure. While the lower sublethal concentration induced cytotoxicity after day 6, the higher sublethal concentration caused cytotoxicity at very early time points (not shown).

From the results obtained, it was quite evident that the higher sublethal concentration caused significant cytotoxicity at early time points and thus did not allow for repeated exposures. The lower sublethal concentration, on the other hand, showed cytotoxicity after day 6. The experimental design was therefore revised to disregard the higher sublethal concentration and the exposure time points beyond day 6 for the lower sublethal concentration. The revised exposure protocol will, however, consider a more detailed approach towards studying the recovery of the cardiomyocytes through regular washout time periods. The cardiomyocytes will be exposed for 3 hours, 2 days, 4 days and 6 days followed by a drug washout until day 14. During the washout period, the medium will be changed every 48 hours. The revised exposure protocol and the sample collections are illustrated below (*Figure 4.28*).

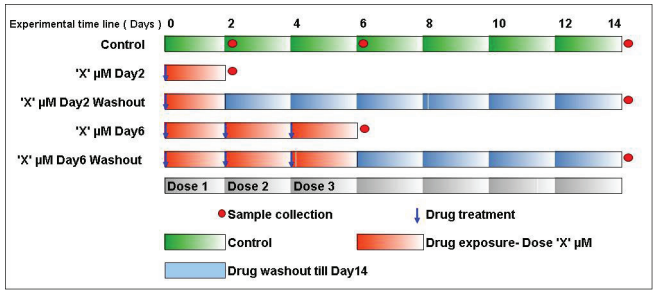

Figure 4.28 *The experimental design and timeline for repeated dose toxicity studies in human iPSC-derived cardiomyocytes. Data source: University of Cologne, Germany.*

This experimental design will be used for transcriptomic, metabolomic, epigenetic and proteomic studies (started but not reported here).

4.4.6 Identification of Biomarkers for Nephrotoxicity

Introduction

The RPTEC/TERT1 represents a state-of-the-art cell line for proximal tubule testing. These cells have been immortalised by introducing the catalytic part of human telomerase into primary human proximal tubule cells, which prevents the cells entering replicative senescence without affecting other differentiated functions (*Wieser et al., 2008*). The cells have functioning p53 and p16 and thus enter cell cycle arrest on contact inhibition, where they then differentiate into a transporting epithelial monolayer (*Aschauer et al., 2013*). The cells have been used to determine the effects of compounds and are particularly applicable to '-omics' techniques (*Wilmes et al., 2013*). We have increasing evidence that the acquisition of stress-response pathways (*Jennings et al., 2013; Jennings, 2013; Wilmes et al., 2011; 2013*) and the loss of differentiated function (*Limonciel et al., 2012*) are highly sensitive mechanistic biomarkers for proximal tubule injury.

Approach

In **DETECTIVE** we want to address the biological effects of repeated dose exposure to compounds in human proximal tubular cells with a view to selecting a panel of markers that are predictive for other toxins. The approach we are taking is to use the highly stable RPTEC/TERT1 cells, which are differentiated for 10 days into a transporting epithelial monolayer tissue prior to use. The cells are then treated with subcytotoxic concentrations of test compounds every day for five days. The effects on the metabolome, proteome, epigenome and transcriptome are analysed at days 1, 3, 5, and after 3 days' recovery. The aim is that the integrated '-omics' analysis identifies pathways that could be used for biomarker selection.

Results

Several functional and '-omics' readouts were employed to assess the effects of compounds on repeated exposure of RPTEC/TERT1 cells. These readouts included impedance, glycolysis rate, quantification of reduced glutathione (GSH) and cellular ATP and transcriptomic analysis.

The xCELLigence system was optimised to allow long-term monitoring of epithelial monolayer integrity during the study. Enhanced glycolysis, as measured by increased supernatant lactate, is a convenient measurement of cell stress (*Figure 4.29*), as it is induced by several common mechanisms including mitochondrial injury and HIF-1 alpha activation and can be employed in repeated dose toxicity regimes (*Limonciel et al., 2011*).

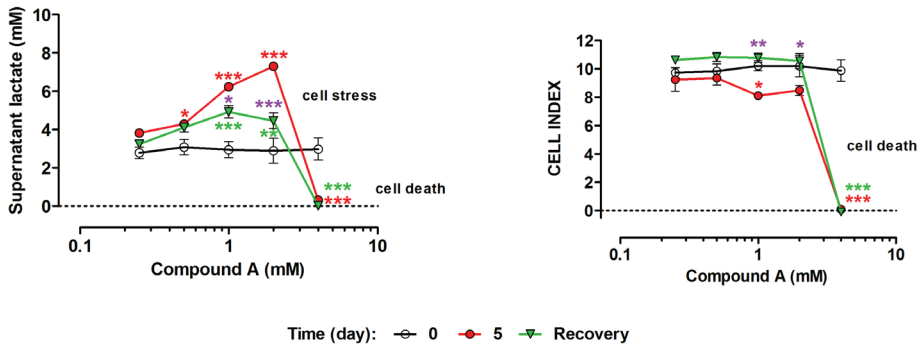

Figure 4.29 *Dose-response effects of compound A in lactate production and impedance (CELL INDEX). RPTEC/TERT1 cells were differentiated on 96 well plates or E-plates for up to 10 days and treated with compounds for 5 days (red line) with a 3-day recovery (green line). Treatment was every day. Lactate was measured in the supernatant medium. An increase in lactate above the control values is considered compound-induced stress, whereas below the control values as cell death. For impedance a fall below control values is indicative of cell stress. Graph shows selected values for clear visualisation. Statistical analysis with two-way ANOVA; red * indicates significance of day 5 to day 0; green recovery to day 0; purple day 5 to recovery. Data source: Innsbruck Medical University, Austria.*

The protocols for the isolation of RNA, protein, microRNA, DNA, metabolites (supernatant and cellular) and paraformaldehyde fixed cells in epithelial cells were successfully optimised during this period. Metabolites and protein can now be extracted from the same cells and microRNA and RNA can also be isolated together. Based on results from the functional readouts and consultation with ToxBank, two compounds were selected for full testing. The results from the transcriptomic analysis show a deep biological impact of compounds with

clear time effects (*Figure 4.30*) and a clear separation of recovery samples (TR) from control and treated samples. Additionally, one of the compounds demonstrated a clear induction of Nrf2-mediated oxidative stress response, whereas the other compound did not (*Figure 4.31*).

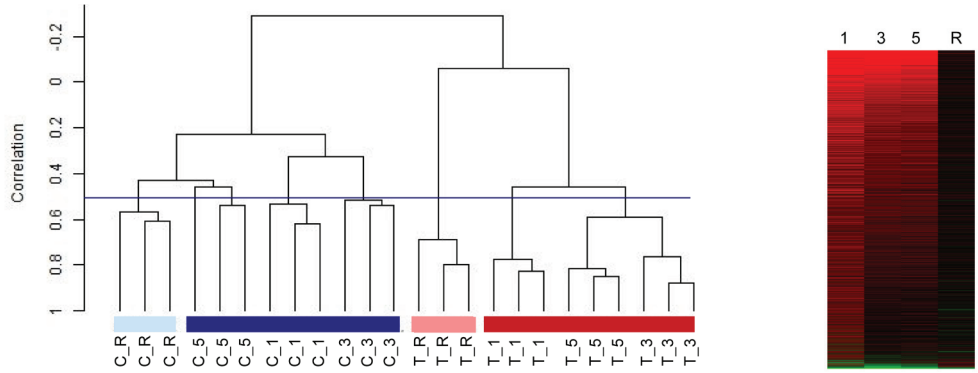

Figure 4.30 *Unsupervised hierarchical clustering of transcriptomic data (left) and a representative heat map of deregulated genes (right). Data source: Innsbruck Medical University, Austria.*

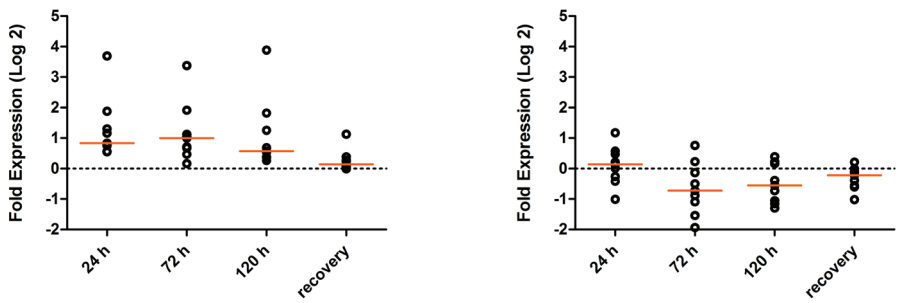

Figure 4.31 *Effect of compound exposure on the deregulation of Nrf2-dependent genes. The circles represent specific Nrf2-regulated genes. The solid line is the median. Data source: Innsbruck Medical University, Austria.*

4.4.7 Innovation

The main innovation of the selected research highlighted in this report includes:

In vitro systems of primary human hepatocytes that can be effectively used for identification of molecular mechanisms of toxicity: Our transcriptomic and proteomic screenings are beginning to reveal hundreds of genes and proteins in primary hepatocytes that are severely affected

by exposure to acetaminophen at a toxic concentration. The biological significance of the identified biomarkers will be determined in the following years. Nevertheless, it is clear that not all aspects of *in vivo* cell physiology are preserved in cultivated hepatocytes. Importantly, we identified a dramatic downregulation of the ER-stress sensor IRE1α in cultivated hepatocytes. This implies that primary hepatocytes are compromised in their ability to sense and react to misfolded proteins. Little is known about mechanisms controlling the expression of ER-stress sensors. We are intensively working on determining the causes leading to IRE1α downregulation, focusing on protein degradation mechanisms due to the remarkably fast decay already observed in freshly isolated hepatocytes. Later we will determine if similar effects are induced on other ER-stress sensors such as PERK and ATF6, and further ER components to determine if this is a general ER adaptation to stress, or if it only affects a small but important component of ER stress sensors.

An increased understanding of the toxic mode-of-action and thus improved liver toxicity prediction in early stages of toxicological screening: For selected biomarker genes, a panel of GFP-tagged HepG2 reporter cell lines that focus on many adaptive stress-response pathways has been constructed. The GFP-fused proteins are controlled by their own promoters, enhancers and other regulatory elements, ensuring the endogenous response to compound-induced toxic effects. The combined stress-response pathway activation and timing will enable a more detailed understanding of the toxic mode-of-action and thus improve toxicity prediction in the early stages of toxicological screening.

A repeat dose exposure protocol designed for long-term toxicity study using human iPSC-derived cardiomyocytes: Our results show that the chosen human iPSC-derived cardiomyocytes can be maintained in good culture conditions for up to 14 days, giving a window for long-term repeated dose toxicity studies. We have employed a comprehensive approach of integrating high-throughput functional and '-omics' assays for assessing the long-term cardiotoxic effects of the drug with repeated exposure. Promising results from transcriptomic and metabolomic analysis show a significant effect on the mitochondrial functioning in a time- and dose-dependent manner, allowing us to focus on this and other pathways affected.

A fully integrated '-omics' analysis for two compounds for the kidney model: The '-omics' methodologies include transcriptomics, metabolomics, proteomics and epigenetics, making this a world-first study in toxicology on integrating epigenetics and more classical '-omics' technologies.

4.4.8 Cross-Cluster Cooperation

The **DETECTIVE** consortium promotes strong collaboration with the other projects of the **SEURAT-1** Research Initiative, aiming to strengthen the efforts of all and to deliver results effectively.

Considering the selection of compounds in the consortium, **DETECTIVE** has always consulted ToxBank for their expert advice. **DETECTIVE** partners German Cancer Research Centre and Quretec have actively communicated with ToxBank partners regarding the activities of the **SEURAT-1** Data Analysis Working Group and on platforms and technologies for sharing of **DETECTIVE** data, respectively.

With regards to the **SEURAT-1** Working Groups (see section 4.10.2–4.10.8), **DETECTIVE** has shown active participation in four of the six working groups. Partners of the **DETECTIVE** consortium are co-leaders in Data Analysis Working Group, Gold Compounds Working Group, Stem Cells Working Group and Mode-of-Action Working Group. **DETECTIVE** has participated in the alpha test of the ToxBank Data Warehouse, which was carried out in July 2012. Our partners have also participated in an online workshop about the toxicogenomic reference database DrugMatrix and the high-throughput screening initiative Tox21, which was organised by ToxBank.

As one part of the **DETECTIVE** database creation involves the creation of a database for '-omics' data, in order to not duplicate the efforts of ToxBank and their ToxBank Data Warehouse for storing such data, **DETECTIVE** will concentrate its efforts on providing effective means of data collection. The data obtained from the **DETECTIVE** partners will be converted to ISATAB format and sent to the ToxBank Data Warehouse.

Lastly, ongoing cluster-level cooperation with NOTOX currently involves discussions of certain characteristic features of primary human liver cells. NOTOX has conducted extensive long-term *in silico* toxicity prediction studies using this particular cell system.

4.4.9 Expected Progress within the Third Year

In 2013, **DETECTIVE** will further define the project roadmap through several workshops and meetings. The roadmap will include a more detailed strategy for the identification of biomarkers of repeated dose toxicity, in order to contribute to the prediction of the onset of several critical 'Adverse Outcome Pathways' (AOPs) in the three target organs. It will also focus the work and determine how to contribute to the **SEURAT-1** proof-of-concepts through the establishment of one or several case studies. The foreseen case studies may consider, but would not be limited to:

- comparison of *in vitro* systems from the same organ for best predictability;
- comparison of '-omics' data generated for *in vitro* systems from different organs for identification of organ-specific and common toxicity signatures;
- verification of 'stress-response pathway'-associated biomarkers;

➠ classification of compounds with respect to ‘stress-response pathway’ activation;

➠ testing of compounds with similar chemical structures.

Furthermore, **DETECTIVE** will continue analysing the functional and ‘-omics’ data generated by the three organ groups during the first two years of the project. The raw data will be checked, pre-processed and analysed in order to provide a high-quality data set for the subsequent biomarker identification. Thorough monitoring of the data quality will ensure that only high-quality raw data will be used for biomarker identification and analysis. The experimental data will also be used to derive endpoints such as EC50, gene expression profiling, etc. The lists of putative biomarkers for a given type of toxicity will be updated based on the raw data analysis and the derived endpoints. The functional context of these biomarkers will be explored using methods from biostatistics and bioinformatics (e.g., pathway analysis), supporting the strategy of establishing AOPs. The relationship between clinical signs and biomarkers will be analysed upon the availability of clinical signs data. In parallel, the *in vitro* systems developed by the three organ groups will be continuously validated through testing of more substances using ‘-omics’ and functional readouts.

All newly generated raw and processed ‘-omics’ and functional data will be fed into the **DETECTIVE** database. In parallel, the newly generated data and the refined experimental protocols will be uploaded into ToxBank. The database will be continuously supplied with biomarkers retrieved from current scientific literature or from currently applied protocols in established pre-clinical safety toxicity studies. This type of information will allow the derivation of *in vitro* biomarkers for repeated dose toxicity testing. By this means, cluster-level interaction will be further improved with mutual benefits for all **SEURAT-1** partners. Additionally, **DETECTIVE** will work towards:

➠ systematic analyses of toxicological effects by high-content imaging techniques for identification of parameter characteristics of defined toxic pathways;

➠ advanced functional analysis of electric properties of *in vitro* cell models based on integration of both multi-electrode microarrays and an impedance system;

➠ refinement of the standard operating protocols for the different ‘-omics’ analyses, especially concerning the optimisation of the preservation of the biological samples’ features;

➠ the analysis of the established reference compounds for additional ‘-omics’ parameters such as global microRNA expression and epigenomic modulation.

Finally, the **DETECTIVE** summer school will be organised in 2013, to transfer knowledge within the consortium, in particular to and between the younger scientists involved. It will also further enhance the already fruitful collaboration between project partners.

4.4.10 Future Perspectives

Successful completion of **DETECTIVE** will change our understanding of repeated dose toxicity testing methods, subsequently leading to a screening pipeline of functional and '-omics' technologies, including high-content and high-throughput screening platforms, to develop and investigate human biomarkers for repeated dose toxicity in human cellular *in vitro* models. Establishment, selection and verification of highly predictive biomarkers in a pathway- and evidence-based approach constitutes a major building block in an integrated approach towards the replacement of animal testing in human safety assessment and will lay the foundation for subsequent efforts in SEURAT-2. The following Research Initiative could address the limited scope of **DETECTIVE/SEURAT-1**, which mainly covers the use of a limited number of human primary cellular systems and test compounds. The employment of several more cellular systems and test compounds and of available human ES/iPS cell-derived systems, and the testing of a more extensive range of toxicological substances, would broaden our knowledge about long-term toxicity. This data expansion and the resulting knowledge will be highly relevant to establishing a solid and reliable basis on which future *in vitro* test systems used by industry can be built. The scientific expertise related to detection of endpoints and biomarkers for repeated dose toxicity, derived by the end of the **DETECTIVE** project, will help to establish a proof-of-concept-based detailed roadmap towards a novel repeated dose toxicity *in vitro* testing platform. This platform should be one aspect of a SEURAT-2 Research Initiative, along with testing and assessing several other human cell systems and establishing high-throughput screening platforms for various drug libraries.

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4.5 COSMOS: Integrated *in Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety

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4.5.1 Introduction and Objectives

There is a desire to be able to obtain information regarding the safety of a cosmetic ingredient directly from chemical structure. Currently computational, or *in silico*, methods to predict toxicity include the use of strategies for grouping (also termed category formation), read-across within groups, (quantitative) structure-activity relationships ((Q)SARs) and expert (knowledge-based) systems. These are supported by methods to incorporate Threshold of Toxicological Concern (TTC) and kinetics-based extrapolations for concentrations that may arise at the organ level (such as physiologically-based pharmacokinetic (PBPK) models).

Currently, these models are simplistic and do not fully capture the repeated dose effects of cosmetics to humans. This is partially a result of insufficient data due to historical and poorly maintained databases as well as the complexity of the endpoint to be modelled. The current knowledge gaps are illustrated and summarised in *Figure 4.32*.

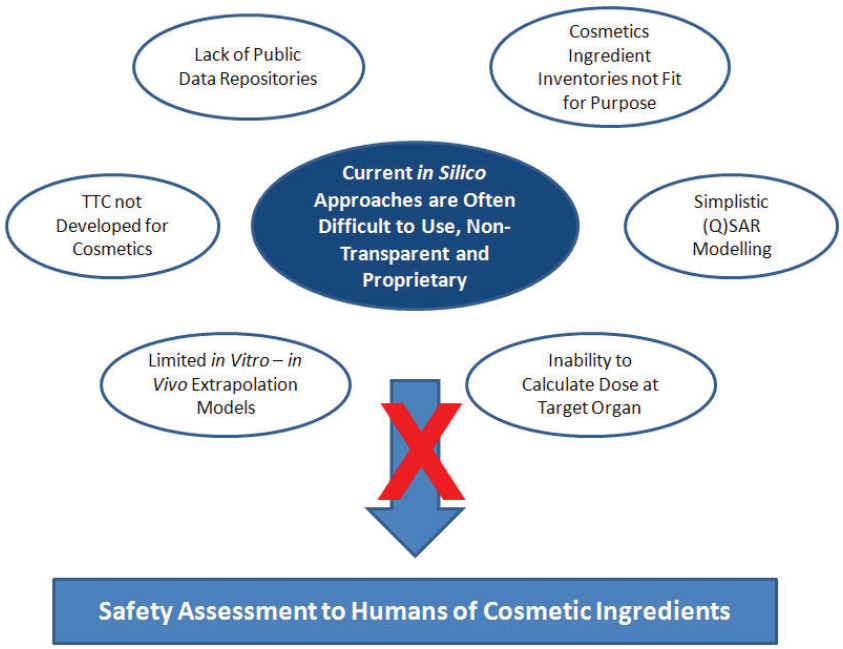


Figure 4.32 *Summary of the knowledge gaps preventing the assessment of the safety of cosmetic ingredients to humans from computational techniques.*

The expectation of a single computational approach to predict the complex series of biological effects underlying repeated dose toxicity to humans is limited as current approaches do not take account of many different mechanisms to enable extrapolation and prediction and are insufficiently supported by data. Therefore, the aim of the **COSMOS** project is to develop synergistic workflows for the prediction of repeated dose toxicity to humans for cosmetics, the integrated use of multiple models being expected to provide an alternative assessment strategy. The *in silico* – open source and/or open access – workflows will integrate models based on the TTC approach, innovative chemistry and physiologically-based pharmacokinetics. This is in line with the current paradigm-shift in toxicology towards developing models based on an understanding of the underlying mechanisms involved in eliciting an adverse effect. They will be adaptable and form a set of building blocks allowing users to incorporate their own data and search existing data compilations.

The specific objectives of **COSMOS** are:

- Collate and curate new sources of toxicological data and information from regulatory submissions and the literature.
- Create an inventory of known cosmetic ingredients and their associated quality controlled chemical structures.

➠ Develop the TTC approach for endpoints relating to human repeated dose toxicity and assess its applicability to cosmetics.

➠ Develop innovative toxicity prediction strategies based on chemical categories, read-across and QSARs related to key events in Adverse Outcome Pathways (AOPs).

➠ Develop a multi-scale modelling approach including cell-based and physiologically-base pharmacokinetic (PBPK) models to predict target organ concentrations and extrapolate from *in vitro* to *in vivo* exposure scenarios.

➠ Use the KNIME technology to integrate access to databases and modelling approaches into adaptable and flexible computational workflows that will be made publicly accessible and provide a transparent method for use in the safety assessment of cosmetics.

Later in this chapter, work undertaken and results achieved regarding biokinetics modelling and toxicity prediction strategies linked to Adverse Outcome Pathways (AOPs) will be described (see sections 4.5.3 and 4.5.4, respectively).

4.5.2 Main Achievements and Challenges in the Second Year

COSMOS Inventory and Database

One of the significant outputs from **COSMOS** has been the work on the compilation of an inventory of substances used or found in cosmetic products, with information coming from the European Commission's Cosmetic Ingredients (CosIng) database and the US Personal Care Products Council (PCPC) lists, after extensive quality control. The combined inventory represents substances of 19,597 INCI (International Nomenclature of Cosmetic Ingredients) names and 9,857 CAS (Chemical Abstracts Service) registry numbers associated with 66 unique use functions. The inventory has been successfully imported into the **COSMOS** database (COSMOS DB), a structure-searchable relational database linking the chemical structure to repeated dose toxicity data. The COSMOS DB structure has been further advanced in the second year, as well as the development of a web application user interface for public access to the **COSMOS** high quality toxicity data. The COSMOS DB can be searched online by chemical structures and other identifiers, as well as by toxicity studies. To capture systemic toxicity, the study parameters considered are study types, species, treatment duration, number of dose levels and non-neoplastic effects. Numeric endpoint decisions such as NO(A)EL (no observed (adverse) effect level) or LO(A)EL (lowest observed (adverse) effect level) values were also captured in the oral toxicity DB. Repeated dose (chronic/subchronic, reproductive/developmental, neuro-, immuno-) toxicity studies have been compiled from existing data sources. **COSMOS** partners also have and continue to harvest oral toxicity data,

applying a systematic quality control process. Sources of data include the US FDA (Food and Drug Administration) PAFA (direct food additives and colourants) and OFAS (food contact substances) databases, US EPA (Environmental Protection Agency) ToxRefDB, European Commission SCCS (Scientific Committee on Consumer Safety) opinions, ECHA (European Chemicals Agency) Registered Substances database and the scientific literature. In addition, further data were donated by a Cosmetics Europe member and **COSMOS** partners. To address the oral to dermal extrapolation of repeated dose toxicity through PBPK modelling, bioavailability data are also needed. To this end, skin penetration and oral absorption data are also being compiled along with dermal metabolism information where available.

A bespoke data entry system has been designed to ensure data quality, following comprehensive reviews of system and user requirements. Moreover, strategies for data curation and data record consistency/reliability assessment have been developed, including the proposal of an innovative approach using fuzzy logic (*Yang et al., 2013*).

To support TTC analysis, an algorithm has been developed and implemented in KNIME (http://www.knime.org) to export selected toxicity data from the **COSMOS** DB to be used as a preliminary basis for the **COSMOS** non-cancer TTC (candidate) dataset. The extraction of TTC data from the oral toxicity database includes a selection process for studies meeting strict criteria and consideration of critical systemic effects. For the final TTC dataset, NOAEL/LOAEL decisions are made by toxicologists.

Threshold of Toxicological Concern (TTC) Approach for Cosmetic Substances

The TTC approach is being expanded for application to cosmetic substances (ingredients and impurities) found in cosmetic products. The compiled initial **COSMOS** TTC dataset has been reviewed extensively (by **COSMOS** ILSI Expert Groups) while being extended with new harvested data by **COSMOS** partners.

The NOAEL/LOAEL values were selected according to the following selection process: For US FDA PAFA and OFAS data, studies were selected according to acceptable study parameters species, oral routes, duration, study endpoint types, and critical systemic effects (excluding neoplastic lesions). To select reliable data records, only studies compliant with FDA Redbook (good laboratory practice (GLP) studies) or well-designed experiments (meeting the core standard by FDA PAFA SOP) if non-GLP were included. US EPA ToxRefDB data were used as provided by EPA. US EPA also applies similar criteria of 'data usability' for GLP and non-GLP studies. For scientific literature data, FDA PAFA SOP for core standard requirements were applied. Data from the ECHA Registered Substances database were selected according to Klimisch scores (reliable without and with restrictions). The NOAEL/LOAEL values from the Munro dataset and US EPA IRIS were included as they were. From these selected studies

across the different sources, the minimum NOAEL value was selected per substance and the accompanying LOAEL was recorded when available. When no NOAEL values were available, the study providing the minimum LOAEL value was selected. The interim **COSMOS** non-cancer TTC v1.5 dataset (650 test substances) was then investigated thoroughly. A portion of the TTC dataset has been under quality assessment (QA) by the **COSMOS** ILSI Expert Group (EG). During the review process, the NOAEL decisions have been made for substances within the lower 10% of the distribution. Also included in this QA process were the studies for the substances whose NOAEL values varied greatly across the different regulatory sources. ILSI EG members rated the studies with the Klimisch score and only 'reliable without restriction' and 'reliable with restriction' were used. NOAELs from regulatory sources were adopted whenever appropriate and available. The data following QA have been combined with the **COSMOS** TTC dataset and the resulting compilation will be available as a database with the NOAEL decision and rationale from the ILSI EG as well as original sources. To continue to ensure data acceptance reliability, **COSMOS** partners have applied the set of review/quality control protocols established by ILSI EG toxicologists to the ongoing oral toxicity data harvesting of chemicals used in cosmetic products.

Furthermore, the relevance of the dermal route of exposure to TTC (bearing in mind its importance for cosmetics) is being examined. Extensive databases of additional dermal and oral absorption data are being curated and reviewed for quality.

Computational Tools for Toxicity Prediction

Workflows have been created that enable relevant chemical categories to be formed, allowing for read-across predictions relevant to chronic toxicity. These rely on the definition of chemical similarity, which can be defined in terms of the mechanism of action and related specifically to the molecular initiating event (MIE). The MIE is the key initial interaction between a chemical and the biological system that results in the initiation of the biological cascade leading to an adverse outcome. Work has focused on grouping chemicals based on the chemistry relating to the formation of a covalent bond between a protein and a chemical. This mechanistic knowledge is useful for developing categories for a range of endpoints relevant to cosmetic ingredients such as hepatotoxicity.

A set of previously published protein binding structural alerts (*Enoch et al., 2011*), re-coded into a KNIME workflow, was used to profile the 4467 chemicals from the **COSMOS** Cosmetics Inventory v1.0, identifying 910 chemicals with the potential to form a covalent bond with a protein. Of these, 837 contained only a single structural alert and 83 contained multiple structural alerts. Further analysis of the mechanistic domain groupings allowed more mechanistically similar categories to be developed.

The need for a concerted effort to collect together information for a mode of action framework is paramount. **COSMOS** has embraced the AOP approach and is looking into organising

mode of action information. A comprehensive evaluation of (Q)SAR models predicting chronic toxicity endpoints, e.g. repeated dose toxicity, and selected target organ/tissue toxicities has been initiated. The search is covering the literature as well as available databases of QSAR models, e.g. the JRC QSAR Model Database.

Toxicokinetics

The computational prediction of biokinetics is vital for successful application of *in vitro* to *in vivo* extrapolation methods.

A cell growth and toxicity model based on Dynamic Energy Budget (DEB) theory has been developed and implemented to simulate High Throughput Screening (HTS) in laboratory toxicity experiments. The model has been used to analyse experiments with 3T3, HepG2, HepaRG and A549 cell assays and it is able to predict the dynamics of cell population as toxic concentration increases. In addition, it provides an estimate of internal concentrations inside the cells, which has been partially validated for polybrominated diphenyl ether (PBDE), methyl mercury and arachlor.

Physiologically-based toxicokinetic (PBTK) models have been proposed. Two degrees of complexity and calibration were distinguished depending on the availability of *in vivo* data. When applied to case studies, model predictions were close to experimental results, even for a model entirely calibrated based on alternative methods (acetaminophen case study).

A model of the liver has been constructed starting from hepatocytes containing a metabolic network model based on cell properties and gene expression. Populations of hepatocytes have then been coupled to a tube-like structure representing the capillaries in the liver, called sinusoids. It was possible to estimate liver clearance, to identify liver vulnerable zones and to predict threshold concentrations for liver failure. The model also permits predictions of genome-based liver responses and adverse effects using different expression levels of cytochromes P450 (CYPs).

Furthermore, new data have been produced relative to effects on liver and metabolism for the cosmetic ingredients coumarin, isoeugenol and benzophenone-2, which share a common metabolism pathway and are likely to be hepatotoxic, alone or in mixtures.

COSMOS KNIME Software and Workflows

The flexible open access platform KNIME is being used to integrate access to the database and application of the models developed within **COSMOS**. Workflows can be shared through the KNIME Desktop client or with a web browser using the Web Portal. The platform has been further expanded in the second year.

A workflow is not a static protocol but usually evolves over time, adding more functionality and/or incorporating new methods and technologies. However experiments should be reproducible, even when the underlying workflow has changed. The needs and technical concepts of workflow archival and retrieval functionalities and their implementation in the KNIME Server have been defined.

COSMOS partners have coded their work into KNIME workflows, e.g. mechanistic profilers, an algorithm to query the database to export the results of toxicity studies and the cell growth and toxicity model based on the DEB theory to simulate high throughput screening in laboratory toxicity experiments.

4.5.3 Selected Highlight I: Category Formation Based on 3-D Molecular Modelling of Receptor Interactions

Introduction

The **COSMOS** Project has adopted the **SEURAT-1** strategy of developing toxicological information with an Adverse Outcome Pathway (AOP) framework. The purpose of this is to organise knowledge regarding how chemical substances may adversely affect human health and to employ this knowledge to develop complementary theoretical, computational and experimental *in vitro* models able to predict endpoints needed for safety assessment. The AOP broadly encompasses and expands upon the knowledge of a mode-of-action (MoA) of a substance. In this context, the MoA describes the sequence of events resulting from the exposure of a living organism to a substance that starts with the interaction of the agent (the substance) with a target biomolecule and results in adverse health effects through functional and anatomical changes, at the cellular level. It has been defined as 'a common set of biochemical, physiological, or behavioural responses that characterise an adverse biological response where major, but not necessarily all, linkages between a direct initiating event and an adverse outcome are understood'. In comparison, a mechanism of action describes such changes at the molecular level and it has been defined as 'a complete and detailed understanding of each and every step in the sequence of events that leads to a toxic outcome', which includes detailed knowledge of the causal and temporal relationships among all the steps leading to a specific effect (*NRC, 2007*).

An AOP is a broader conceptual construct that describes pathways initiated via non-specific interactions, as well as more specific ligand-receptor interactions leading to adverse outcomes (*Ankley et al., 2012*). The pathway approach is based on the concept that toxicity results from the substance first reaching and then interacting with an initial key target in the organism. According to this theory AOPs are generally a sequential series of events that, by definition, span multiple levels of biological organisation. The content connecting the initiating event to the outcome can take various forms, depending on the types and extent of biological information

from which the AOP is derived or the risk context that it is being used to address. The AOP concept requires an anchor to both a molecular initiating event and an adverse outcome with significance to risk assessment (*Cronin et al., 2010*).

The report published in 2012 within **SEURAT-1** entitled 'Description of Prototype Modes-of-Action Related to Repeated Dose Toxicity' presents the definition and detailed documentation of chosen toxicological MoAs associated with repeated dose target organ toxicity as a first step in building a 'prototype' safety assessment framework (*Landesmann et al., 2012; OECD, 2012*). The two chosen MoAs related to chronic liver toxicity are namely 'MoA from Protein Alkylation to Liver Fibrosis' and 'MoA from Liver X Receptor Activation to Liver Steatosis'.

COSMOS supports the development and promotion of adverse outcome pathways by organising the chemistry involved in the processes. One of the approaches selected within **COSMOS** to understand the chemical toxicity in the MoA/AOP framework is molecular modelling to study the binding of small molecules (the toxicants) to biological macromolecules (biological receptors/targets) applicable when the MoA of interest includes receptor binding as a key event. This is the case of the 'MoA from Liver X Receptor (LXR) Activation to Liver Steatosis' where the molecular initiating event is the binding to LXR and the activation by appropriate ligands.

LXRs are ligand-activated transcription factors that belong to the nuclear receptor superfamily of hormone. Upon activation LXRs form heterodimers with retinoid X receptor (RXR) and bind to the LXR response element found in the promoter region of the target genes. LXR activation regulates the expression of key target genes that are involved in cholesterol metabolism and transport, glucose metabolism, and inflammation. Thus, LXR agonists show beneficial effects in animal models of antiatherosclerosis, diabetes, inflammation, and neurodegenerative diseases. However, LXR agonists also increase the expression of genes that control hepatic lipogenesis, such as sterol regulatory element-binding protein 1c (SREBP-1c) and the carbohydrate responsive binding protein. Thus, the beneficial effects of LXR agonists are compromised by the increase plasma and hepatic triglyceride levels resulting in liver steatosis (*Baranowski, 2008*).

There are two LXR isoforms, LXRα and LXRβ, which differ substantially in their tissue distribution: LXRα expression is restricted to liver, kidney, intestine, fat tissue, macrophages, lung, and spleen and is highest in liver. LXRβ is expressed in almost all tissues and organs. The different pattern of expression suggests that LXRα and LXRβ have different roles in regulating physiological function and consequently the role of the two isoforms in relation to adverse effects could be different: selective LXRβ activation may be expected to reverse atherosclerosis, while having no or little effect on hepatic LXRα-dominated lipogenesis. On the other hand LXRα/β antagonists could down-regulate the SREBP-1c pathway to reduce triglyceride levels in hypertriglyceridemic patients without affecting cholesterol homeostasis (*Jakobsson et al., 2012*).

Approach

Molecular modelling techniques have been used in the drug discovery process for more than 50 years to provide useful insights and new suggestions for molecular structures to be synthesised, and cost-effective (virtual) experimental analysis prior to synthesis. Modern drug discovery, in fact, involves the identification of screening hits and the optimisation of those hits to increase their affinity, selectivity (to reduce the potential of side effects), efficacy/potency, metabolic stability (to increase the half-life), and oral bioavailability. One or more of these steps may involve molecular modelling techniques. Typically a drug target is a key macromolecule, a biological receptor, involved in a particular metabolic or signalling pathway that is specific to a disease condition or pathology or to the infectivity or survival of a microbial pathogen. Some drug discovery approaches attempt to inhibit the functioning of the pathway in the diseased state by causing a key biological target to stop functioning. Small molecules are designed to bind to the active region and inhibit this key biological target.

As the interaction between a small molecule with a biological target is a key step in some mechanisms of toxicity, in principle molecular modelling techniques can be employed in the risk assessment process. Obviously, applying approaches developed for drug discovery to the problem of predicting the potential toxicity of chemicals requires an optimisation of the methods that takes into account differences and similarities between the two contexts.

Where drug discovery needs a sieve, risk assessment needs a magnifying glass. In fact, one of the main aims of drug discovery is to explore the entire chemical space in order to extract a molecule with a given pharmacological activity and a desired ADMET (Absorption, Distribution, Metabolism, Excretion, Toxicity) profile. On the other hand, one of the main aims of risk assessment is to evaluate the effects of a specific molecule on biological systems. Moreover a drug discovery initial screening is tailored to minimise the false positive rate whereas a risk assessment initial screening is designed to minimise the false negative rate. Within drug discovery the most potent binders of the biological target are to be identified minimising the number of ‘false positive’ (i.e. molecules that are predicted to bind correctly to the receptor and accordingly are predicted to be active but that do not show the desired biological activity). Conversely, the general purpose of an initial screening in risk assessment is to identify chemicals that show the toxic activity, active chemicals, while minimising the number of chemicals that are wrongly predicted as non toxic, i.e. false negatives. A feasibility study is reported here where LXR is analysed in order to verify whether it is suitable for molecular modelling approaches.

Results

A comprehensive literature search was performed in order to retrieve experimental information on available 3-dimensional models of LXR and previous molecular modelling studies on this receptor. A preliminary molecular modelling study was also carried out (see section 4.9.3.2).

As lipid-activated nuclear receptors, LXRs are composed of a highly conserved DNA binding domain (DBD) and a ligand binding domain (LBD), which can be targeted by endogenous ligands, as well as by synthetic ligands. 17 3D models of the LBD of LXR were retrieved by means of a search in the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB http://www.rcsb.org/pdb/home/home.do; *Table 4.1*). All models were X-ray crystallography determined structures of complex assemblies of the LBD of LXR, a co-crystallised ligand and a co-crystallised protein. The secondary structure of the LBD of LXR, formed by 12 α-helices creating a mainly hydrophobic ligand binding pocket (LBP), is highly conserved through the LXR structures of both subtypes.

Table 4.1 *3D models of the LBD of LXR available at the PDB (accessed 15 February 2013).*

PDB ID	Ligand	Cocrystallised proteins
LXRα, *Mus musculus*		
2ACL	synthetic agonist SB313987	dimer with hRXRα
3FAL	synthetic agonist GSK2186	dimer with hRXRα
3FC6	synthetic agonist SB786875	dimer with hRXRα
LXRα, *Homo sapiens*		
1UHL	synthetic agonist T0901317	dimer with hRXRβ, synthetic coactivator
3IPQ	synthetic agonist GW3965	homodimer, synthetic coactivator
3IPS	synthetic agonist	homodimer, synthetic coactivator
3IPU	synthetic agonist	homodimer, synthetic coactivator
LXRβ, *Homo sapiens*		
1UPV, 1UPW	synthetic agonist T0901317	monomer
1PQC, 1PQ9	synthetic agonist T0901317	homodimer
1PQ6	synthetic agonist GW3965	homodimer
3L0E	modulator	homodimer
3KFC	synthetic agonist	homodimer
1P8D	sterol agonist	homodimer, synthetic coactivator
4DK7	full agonist	homodimer
4DK8	partial agonist	homodimer

The Basic Local Alignment Search Tool (BLAST http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to carry out a homology analysis of the proteins. The sequence of the human LXRα was compared with the sequence of the murine LXRα. A high degree of homology (98% of the LBD) was calculated. Then the sequence of the human LXRα was compared with the sequence of the human LXRß. The two LBDs have a high degree of homology (78%) that is

highest when considering just the ligand binding pocket (LBP), the region where the ligands interact. This homology analysis indicates that, whereas molecular modelling is suitable for studying LXR binding potency, it is not suitable for modelling LXRα/ß subtype-selectivity.

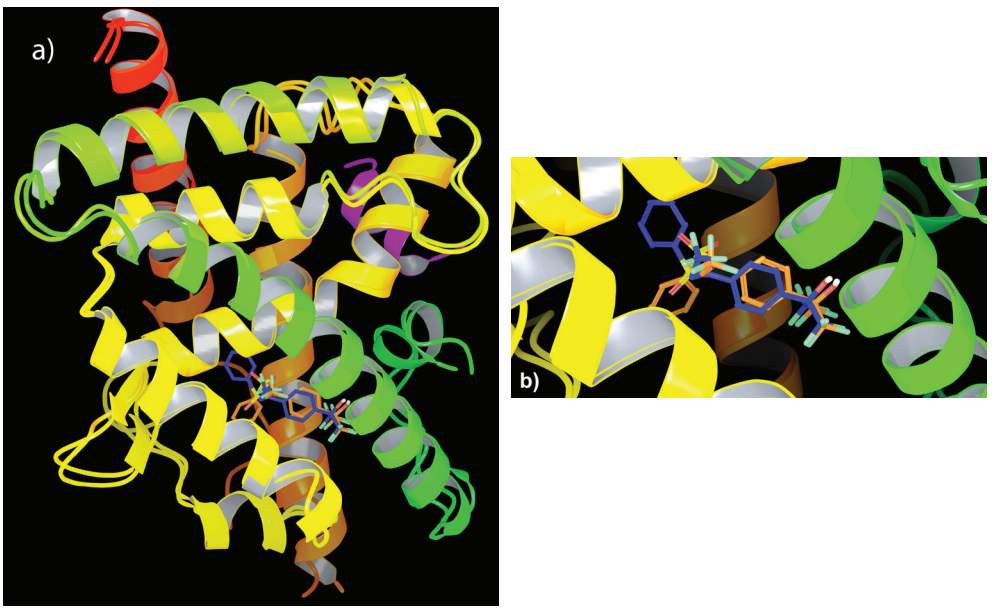
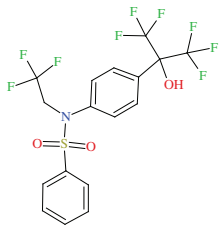
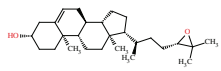
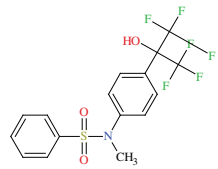
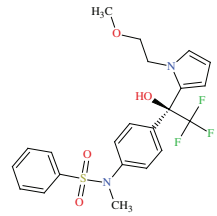
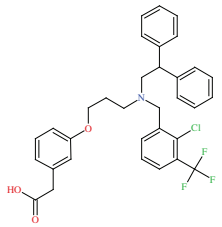
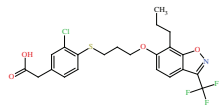
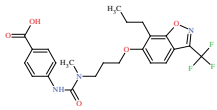
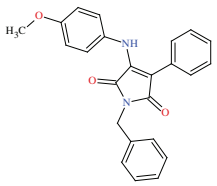
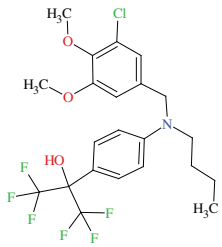
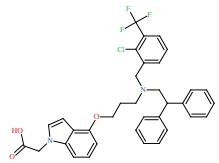
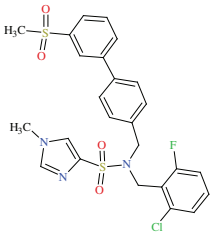
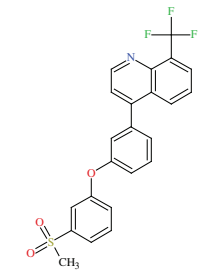


Figure 4.33 *a) Alignment of the ligand binding domain of LXRα/β co-crystallised with T0901317; b) Details of the ligand binding pocket.*

In fact, the superimposition of the crystal structures of LXRα/β co-crystallised both with T0901317 (*Figure 4.33*), the LXR agonist proposed as the reference chemical for liver steatosis by the **SEURAT-1** Gold Compound Working Group, and GW-3965 revealed no outstanding differences in the 3D models of the LBP. Despite a different binding pose of the ligand, the primary, secondary and tertiary structure of the LBP is conserved between the two subtypes co-crystallised with the same ligand. On the other hand, a comparison of the same subtype of LXR bound to different ligands revealed a large plasticity of the LBP to accommodate compounds with noticeably different shapes and sizes (*Table 4.2*).

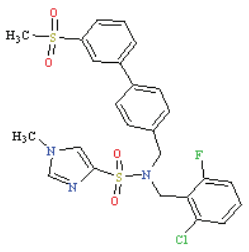
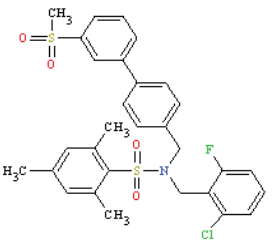
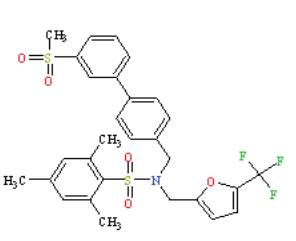
Table 4.2 *Structures of the ligands co-crystallised in the LBDs of LXRα and LXRβ.*

			
Ligand: T0901317	PDB code: 1p8d	PDB code: 4dk7	PDB code: 4dk8
			
Ligand: GW3965	PDB code: 3ips	PDB code: 3ipu	Ligand: SB313987
			
Ligand: GSK2186	Ligand: SB786875	PDB code: 3l05	PDB code: 3kfc

Currently known LXR ligands can be classified into two categories with respect to transactivational action, i.e., those that activate expression of the target genes and those that repress expression of the target genes. They can be defined respectively as transactivational agonists and transactivational antagonists. It seems that the activities of LXR ligands depend upon their effect on recruitment of cofactors to helix 12 of the LXRs: many transactivational agonists induce dissociation of co-repressors whereas many transactivational antagonists induce a conformational rearrangement of helix 12 leading to stabilising the binding of co-repressors (*Albers et al., 2006*). An example of how the agonist/antagonist action can be modulated by slight changes of the ligand chemical structures is shown in *Table 4.3* (*Zuercher*

et al., 2010). Moreover it seems that the ligands LXR binding affinity is not correlated with their transactivational action (pIC_{50} values in *Table 4.3*). Considering that: (i) the binding site seems to be the same for both agonists and antagonists; (ii) molecular modelling is designed to calculate the interactions between the receptor and the ligands and hence mainly the binding affinity; (iii) the binding affinity is not correlated with the transactivational action; (iv) the differences in the chemical structures of agonists versus the antagonists ones are negligible; molecular modelling will be employed to address LXR binding and not agonism/antagonism action.

Table 4.3 *Structures and binding affinity of an agonist and an antagonist of LXRβ.*

		
pIC_{50} = 8	pIC_{50} = 7.8	pIC_{50} = 7.5
Agonist	Partial Antagonist	Antagonist

As the products of the LXR target genes are involved in lipid metabolism, reverse cholesterol transport, and glucose transport, it is thought that LXRs are promising drug targets for treatment of atherosclerosis, hyperlipidemia, or metabolic syndrome. Several molecular modelling approaches, such as docking, virtual screening (*Ghemtio et al., 2010*) and 3D pharmacophore analysis (*Grafenstein, von et al., 2012; Zhao, 2011*), have been employed for more than 10 years to design drugs able to bind to LXRs. Moreover several 2D and 3D QSAR (quantitative structure-activity relationship) models predicting LXR binding affinity (expressed as IC_{50} values toward the b isoform) are available in the literature. These QSARs are mainly based on congeneric series of different LXR ligands, e.g., podocarpic acid analogues, quinolines and cinnolines, 1H-indol-1-yl tertiary amines, etc. Different modelling approaches are used and, in some cases, combined, including molecular docking, multidimensional QSAR, fragment-based QSAR and 3D-pharmacophore modelling. The majority of the models are externally validated, showing good predictive performance, but they are characterised by a limited applicability domain.

4.5.4 Selected Highlight II: Prediction of *in vivo* Hepatotoxic Effects Based on *in vitro* Data and Modelling: *A* Case Study with *A*cetaminophen

Introduction

In the context of replacement of animal testing, it is expected that most of the toxicological information will be obtained through structure-activity models and *in vitro* assays on human cells or cell lines. However, most of the toxicological information for a chemical obtained through *in vitro* testing provides information on the dose response relationship at target cell or, more rarely, at target organ level. To integrate the *in vitro* results into quantitative *in vivo* risk assessment, it is thus necessary to relate the toxicokinetics (TK) of the chemical in the body and the toxicodynamics (TD) at each target level (*Andersen & Krewski, 2010; Adler et al., 2011; Louisse et al., 2012; Yoon et al., 2012*). This can be achieved through Physiologically-Based Toxicokinetics or Pharmacokinetics models – PBTK or PBPK (*Clewell et al., 2008*). A PBTK model consists of a series of mathematical equations which, based on the specific physiology of an organism and on the physicochemical properties of a substance, are able to describe the absorption, distribution, metabolism and elimination (ADME) of the compound within this organism. The solution of these equations provides the time-course of the parent compound and its metabolites in the organs and allows for a sound mechanistic description of the kinetic processes including accumulation in tissues.

There are only a few examples of PBPK models being used to extrapolate *in vitro* response to *in vivo* hazard assessment. *Punt et al. (2011)* proposed a small number of examples where PBPK models have been used to extrapolate *in vitro* toxicity results to the *in vivo* situation. They show that predictions of *in vivo* effects based on integrated *in vitro* and PBPK modelling approaches are generally within one order of magnitude of the observed *in vivo* data. There are, thus, improvements to be made both in the experimental field and in the accurate calibration of toxicokinetics models. *Yoon et al. (2012)* reviewed different quantitative *in vitro* to *in vivo* extrapolations (QIVIVE). They identified key elements that have to be predicted for a successful extrapolation: (i) intestinal absorption and pre-hepatic clearance, (ii) hepatic and extra-hepatic metabolic clearance, (iii) renal clearance, (iv) volume of distribution (for acute *in vivo* exposures). They also indicated that only rare examples of QIVIVE do not rely, at least partly, on *in vivo* data.

Another important aspect is the relevance of the effects (or toxicodynamics) description in the PBPK models. Firstly, to fit the predicted time-course of the concentration at target level, TD models should account for the time dependence of effects, and focus not only on endpoints such as maximum concentration and area under the curve (AUC). Secondly, TD models should account in a realistic way for the physiological context of the effects. In this framework it is necessary to develop physiological models able to capture the interplay between different structures in tissues, organs, and the whole body. The liver is an important organ in this

workflow because it is required for many metabolic functions. This fact has boosted the development of virtual liver models within the framework of large-scale research programmes. Prominent examples are the virtual liver project (*Wambaugh and Shah, 2010*) and the virtual liver network (*Holzhütter et al., 2012*).

The advantage of such approaches is the possibility of predicting substance extraction and distribution that also depends on the heterogeneity of the liver micro-structures (*Hunt & Ropella, 2010*). In several organ models, relatively simple individual cells are coupled to a complex description of the liver (*Kuepfer et al., 2012*). For the prediction of the function and structure of the liver, such coarse grained approaches provide essential information on the physics (behaviour of a granular medium), the way the liver responds to damage, and on the detoxification and drug elimination of this organ (*Chelminiak et al., 2006*). Examples are models where cell populations are described as multi-agent systems ordered in complex networks of the parenchymal tissue (*Chelminiak et al., 2006; Hoehme et al., 2010*). However, a detailed description of the metabolic and regulatory networks is necessary to understand liver function, in particular for the prediction of the effects of drugs (and other substances) in pharmaceutical research (*Kuepfer et al., 2012*). In this field, only a few models have recently taken steps towards the integration of detailed cell mechanisms (*Ohno et al., 2008*), a topic which is also addressed in the **SEURAT-1** project NOTOX (see section 4.6). For instance, there are changes in the distribution of oxygen and metabolites inside the liver introducing a zonation that affects function (*Allen et al., 2005*) as well as cell death in response to toxic doses (*Malhi et al., 2010*). An additional advantage of the incorporation of detailed dynamic cellular models is the possibility to include inter-subject variability in predictions of drug effects (*Bucher et al., 2011; Niklas et al., 2013*).

Approach

We propose a modelling framework to predict *in vivo* liver toxicity (*Péry et al., 2013*). It is composed of a PBPK model to relate dose of exposure and concentration in the liver, a simple dynamic model to analyse *in vitro* concentration-liver cell viability data with time, extended to a much more biologically and physiologically relevant 2D liver model. In a case study, this modelling framework was applied to humans exposed to acetaminophen (APAP), which is a pharmaceutical known for its hepatotoxicity, with available information on doses leading to liver failure.

To assess the relevance of this framework relative to the use of alternative methods for the calibration process, the human PBPK model was calibrated in three different ways: (i) with *in vitro* and *in silico* methods alone, (ii) by extrapolation to humans using a PBPK model calibrated for rats, (iii) or by using all relevant available data, including actual human data. The alternative methods we used were either QSAR models (to estimate octanol-water partition coefficient (K_{OW}), pKa, human intestinal absorption rate, fraction unbound, partition

coefficients and bioavailability) or *in vitro* data for metabolism, accounting for the two main routes of metabolism for acetaminophen (sulfate and glucuronide conjugations). We first analysed *in vitro* data (cell viability) through the calibration of a simple model relating exposure concentration and the decrease of cell numbers per unit of time. We then coupled the PBPK models and the toxicodynamic model to calculate the expected dose response relating *in vivo* liver cell viability long after absorption of acetaminophen and the dose absorbed (*Figure 4.34*).

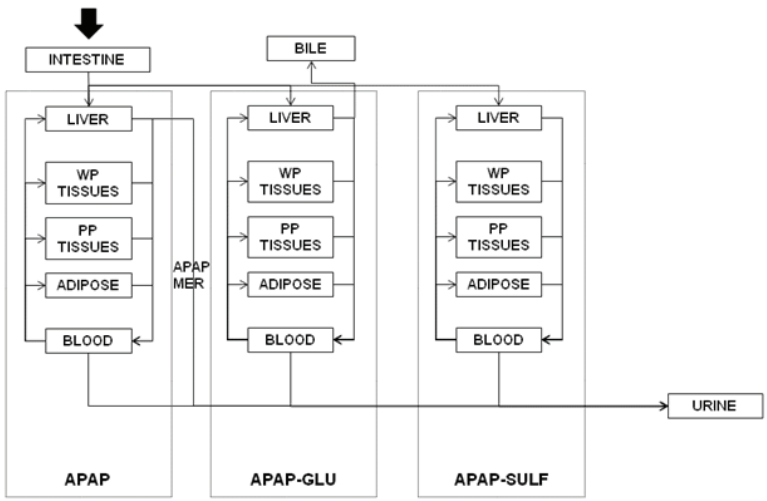


Figure 4.34 *PBTK model used to describe the kinetics of distribution of acetaminophen (APAP) and its main metabolites (glucuronide (APAP-GLU), sulfate conjugate (APAP-SULF)).*

We then modelled the toxic effects in a 2D liver after an overdose of acetaminophen. The primary goals were: (i) to set up and verify a whole body model coupled with an *in silico* liver, (ii) to predict the distribution of acetaminophen and other substances in the whole body; and (iii) to extrapolate critical doses from *in vitro* data. Also, a central issue in this study was the simulation of cell mortality when acute toxicity takes place. To this end, we reconstructed a mathematical network for acetaminophen metabolism in the cell (represented by ordinary differential equations), where three different main modules were simulated: drug metabolism, glutathione (GSH) metabolism and the representation of the synthesis of reactive oxygen species (ROS). If the ROS concentration exceeds a critical value and GSH is consumed, the probability of necrosis for the hepatocytes increases.

A basic assumption underlying this study is that the primary toxic effect is mainly induced by cellular mechanisms. For this reason, we set up and applied an alternative model with a reduced complexity of the liver, suitable for coupling hepatocytes and whole body. Once the cell metabolism was defined, it was integrated into a model for the liver. After the blood enters

the liver, it is distributed by portal veins into functional subunits, called lobules, which carry out diverse functions including the detoxification of xenobiotics at the cellular level (*Figure 4.35*). The distribution of the substance was modelled assuming that it diffuses along this capillary. In the periphery of each lobule, several blood vessels deliver the blood flow into the lobule through additional substructures called sinusoids. The sinusoid was essentially modelled as a collection of hepatocytes aligned along a blood vessel with geometry similar to a cylindrical tube (*Ohno et al., 2008*). Since the central goal of this study was to model drug metabolism in the liver taking place in the hepatocytes, we defined the sinusoids as simple capillary structures with a wall composed only of hepatocytes.

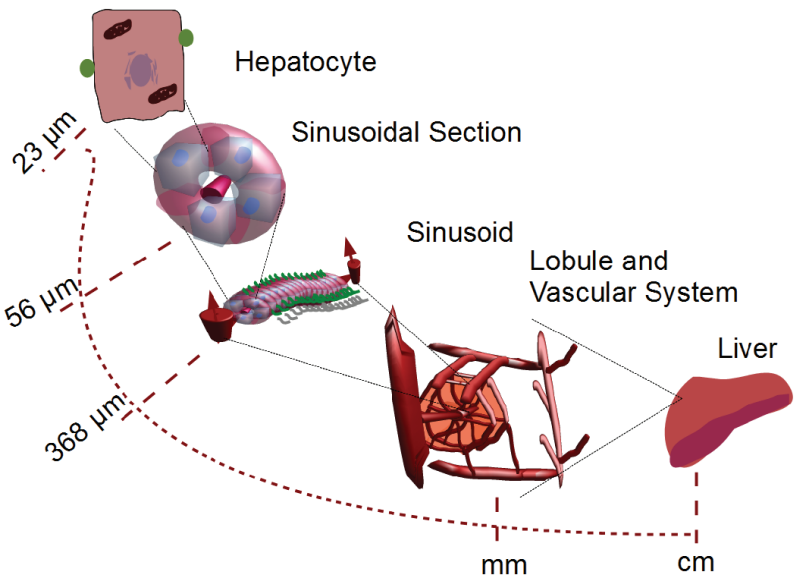


Figure 4.35 *Multi-scale system from single hepatocytes to organ level. Single hepatocytes are coupled to liver capillaries (sinusoids) which are coupled to micro-organelles called lobules. These lobules are considered to be the smallest functional micro-structure in the liver.*

Furthermore, since the cells in the centrilobular region have a low oxygen intake (*Allen et al., 2005*), we assumed that the cells in the respective region are more sensitive to toxic effects compared to cells near the portal veins (zonation). This means that the probability of cell death via necrosis is inversely proportional to the distance to the central vein (*Gujral, 2002*). Finally, the estimation of realistic microdosimetry requires the computation of the distribution of the substance in the whole body. To this end, we replaced the liver compartment in the PBPK model with the multi-scale model of the liver, which is able to transform and remove the substance from the blood (clearance).

Results

The estimated PBPK parameters (calculated using the different above-mentioned calibration methods) differed mainly relative to the estimates of metabolism. Clearly rats and humans show qualitative and quantitative differences relative to metabolism. Mice may be a more relevant model than rats to understand the mechanisms of acetaminophen overdosing in humans. As for humans, in mice sulfation is reduced compared to rats and there is no sulfate depletion at concentrations at which toxicity occurs. Therefore, the *in vitro* value often underestimated the maximum velocity.

Excretion is overestimated through alternative methods (based on filtration rate and unbound fraction in blood). The difference may be due to acute renal toxicity, which is characterised by cellular injury primarily confined to the proximal tubule and significant reductions in glomerular filtration rate.

As for the other parameters, QSAR predictions performed well. The estimated absorption rate was closer to the *in vivo* human estimate than that based on the extrapolation from rats. The estimated fraction unbound to protein (fup) and K_{ow}, which have been used to calculate partition coefficients, could be used to obtain similar partition coefficients between all methods.

Figure 4.36 shows the data and the model for HepaRG cells exposed to acetaminophen as well as the expected dose-response for the three different PBPK-TD models, i.e. a PBPK model calibrated on human data, a PBPK model extrapolated from rats, a PBPK model calibrated on *in vitro* and QSAR data only, which do not differ substantially. If we calculate which dose would lead to a significant difference compared to a control, we obtain 820, 690 and 500 mg/kg for the human data-based, the rat data-based and the alternative methods-based PBPK/TD models, respectively. These estimates are close to the usual range of human overdose, which is between 150 and 500 mg/kg.

The small difference between predictions based on the PBPK models calibrated on alternative methods or human data could be explained by the fact that the *in vitro* underestimation of the metabolism rate was partly compensated by the overestimation of the renal excretion. We performed additional simulations setting the renal excretion value in the PBPK model (calibrated based on *in vitro* and *in silico* data) to the same value as the two other calibrated models. In this case, for a given cell viability, the corresponding dose would have been divided by a factor of 3 (i.e., the dose at which significant effects on *in vivo* cell viability would be expected was estimated at 155 mg/kg).

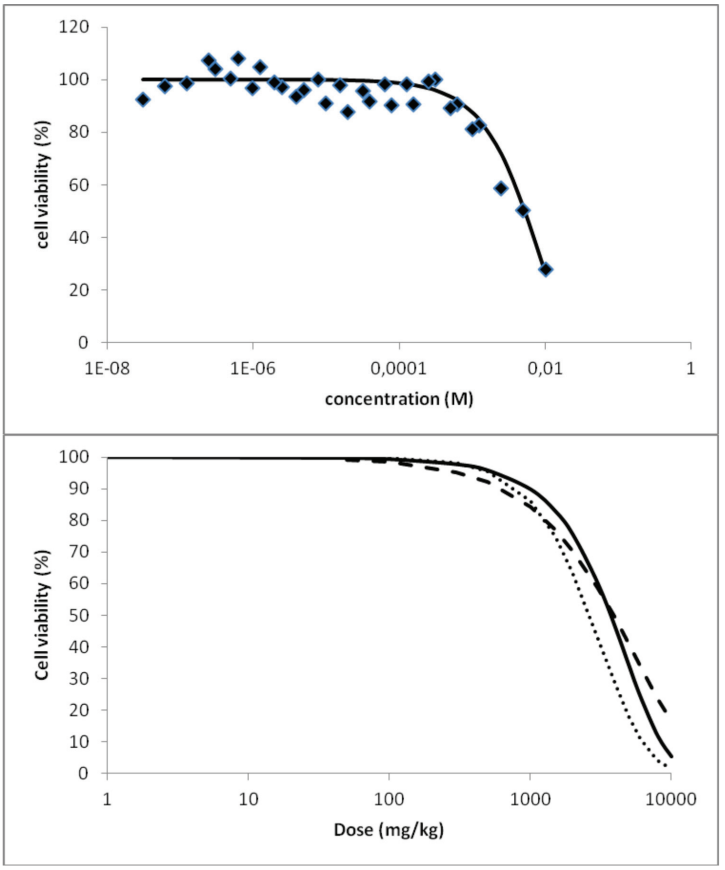


Figure 4.36 *Upper: HepaRG cell viability as a function of acetaminophen exposure concentration. Points represent the actual data and the line represents the regression of the data with our model. Lower: Predicted in vivo cell viability as a function of dose for the three different PBPK-TD models (solid lines: PBPK model calibrated on human data; dotted lines: PBPK model extrapolated from rats; dashed lines: PBPK model calibrated on in vitro and QSAR data only).*

According to the 2D liver model, acetaminophen is distributed from the portal to the central vein showing a rapid transport of APAP in the periportal region. Accordingly, toxic substances should accumulate in the pericentral region, making the cells in this region more prone to APAP toxicity. In *Figure 4.37* the cell population on the liver lobule is represented as a function of time. The uniform orange surface represents the undamaged population of hepatocytes, and the red surfaces represent the six portal veins around the organ. When the concentration of toxic metabolites increases up to a critical level, hepatocytes initiate a necrotic process. Necrosis is represented as a black surface.

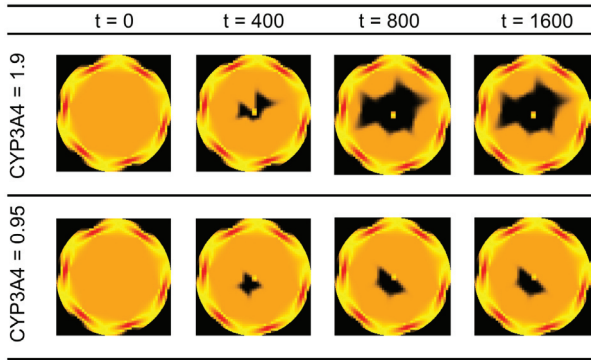


Figure 4.37 *Cell viability in the lobule at different time points (in min) for two different CYP3A4 activities after a single oral APAP dose of 393 mg/kg. Viable cells are schematically illustrated in orange, dead cells in black.*

An advantage of accounting for kinetics at the cellular scale is the possibility to include inter-individual differences in gene expression or enzyme activities into the estimation of drug effects and toxicological risk. The spatiotemporal estimation of cell viability in the liver lobule depending on the activity of CYP3A4, and after a single dose of acetaminophen, is shown in *Figure 4.37*. Given that the definition of the necrosis process is essentially non-deterministic, the degradation of the cells in the lobule is non-symmetric. We also observed that an increase in CYP3A4 activity strongly increases the risk of APAP-induced hepatotoxicity. From this result, two individuals with different CYP3A4 expression factors show different toxic responses to acetaminophen. In general we found that, for the estimation of critical doses, there is a dependency between dose and CYP3A4 expression.

Additionally, the inclusion of necrosis together with the defined liver clearance implies a continuous feedback between local cell behaviour, clearance from the organ, and distribution of the substances in the whole body. We conclude that cell mortality implies a gradual diminution of the capacity of the organ to eliminate APAP. Simultaneously, this leads to an increase in the APAP concentration in the liver, an effect that can exacerbate the toxic response of the organ.

4.5.5 Innovation

Category Formation Based on 3-D Molecular Modelling of Receptor Interactions: Within the MoA/AOP framework **COSMOS** is developing an innovative approach employing molecular modelling techniques for MoAs that involve receptor binding as a key event. This is the case of the 'MoA from Liver X Receptor Activation to Liver Steatosis', one of the toxicological processes associated with repeated dose target organ toxicity chosen by **SEURAT-1** to be defined and documented as a first step in building a 'prototype' safety assessment framework

(see section 4.10.5.2). LXR appears to be a promising case study as it is suitable to be studied with a wide range of molecular modelling techniques, which range from ligand to structure based approaches. Moreover, molecular modelling drug design success stories on LXR are reported in the literature. Though drug discovery and risk assessment share the same key event, the binding to LXR in this case, the different context requires an optimisation of the methods that takes into account differences and similarities between the two. The differences between drug discovery and risk assessment were analysed to provide a valid basis for the future optimisation of the methods to be applied to LXR.

As a first step a comprehensive literature search was performed in order to retrieve experimental information on available SAR data and 3-dimensional models of LXR. The existing information and models were retrieved and analysed. According to this analysis, molecular modelling approaches will be applied to address LXRβ binding affinity. On the contrary neither LXRα/β subtype-selectivity nor agonism/antagonism action will be addressed.

Prediction of *in vivo* Hepatotoxic Effects Based on *in vitro* Data and Modelling: Even with the simple TD model, effects were deduced from concentration at organ level in a dynamic way (a differential equation) accounting explicitly for the time-dependence of the occurrence of adverse effects. Usually, in TK/TD approaches, *in vivo* and *in vitro* effects are related through the area under the curve (AUC) of the parent or metabolite concentration or their maximum concentration. Using a mechanistic and dynamic model to relate cell exposure concentration and cell death may provide more biological relevance. In particular, the extrapolation between different exposure durations or from one dose to repeated doses becomes straightforward.

Another key issue is the possibility to calibrate relevant toxicokinetics models only based on *in vitro* and *in silico* data. This was possible for acetaminophen because it is close, structurally, to chemicals for which QSAR models have been designed (mainly drugs) and because its elimination mainly occurs through urine, while absorption is mainly oral. It is essential to extend our proof of concepts to other categories of substances and to include in a relevant way other routes of absorption and elimination. This is planned to be achieved through the **SEURAT-1** Biokinetics Working Group and direct interactions between **COSMOS** and other **SEURAT-1** projects, mainly with NOTOX.

A central innovation here is the possibility to track the spatio-temporal distribution of substances in the organ and to predict toxic effects. Since we integrated a mechanistic, kinetic cellular model, we were able to analyse how individual properties – such as specific gene expression – affect pharmacokinetics and toxic outcomes. This aspect, together with the representation of a heterogeneous population of hepatocytes (zonated liver), is an advantange that allows an individualised prediction of toxic effects in humans.

Additionally, this method allows the inclusion of physical parameters (such as blood flow, lobule size, diffusion, and dependence on temperature etc.) and important physiological properties

(e.g., zonation of metabolic functions of organs and tissues). In summary, the effects of organ structure and physiology on cellular function can be taken into account, aspects which can hardly be analysed with other, similar models or approaches.

4.5.6 Cross-Cluster Cooperation

COSMOS partners are involved in cross-cutting activities of the **SEURAT-1** Research Initiative, for example the Gold Compound selection Working Group and Data Analysis Working Group, as well as the Mode of Action and Biokinetics Working Groups, the latter co-lead by **COSMOS**.

The overarching cross-cutting activities regarding adverse outcome pathways (AOPs) are a major theme within the **SEURAT-1** Research Initiative. **COSMOS** supports the development and promotion of AOPs, specifically by organising the chemistry involved in the process. **COSMOS** organised the Symposium 'A Roadmap to Navigate from Databases to Adverse Outcome Pathways', held on 12 September 2012 at the University of Bradford, England. PBPK modelling/biokinetics can be used to determine the internal exposure (dose at target organ level) necessary for eliciting the effect. **COSMOS** will thus help to identify highly targeted *in vitro*/*in chemico* assays that could be developed and used to provide evidence to support the pathways.

COSMOS is also involved in the **SEURAT-1** training task force and contributed to shaping the programme of the **SEURAT-1** Summer School 2012 and is discussing the next Summer School in 2014. **COSMOS** has contributed with four training sessions and one plenary lecture on **COSMOS** topics to the **SEURAT-1** Summer School held on 4-8 June 2012 in Oreias, Portugal, which was also open for attendees external to the **SEURAT-1** cluster. The **COSMOS** sessions were attended by **COSMOS** and other **SEURAT-1** members as well as external attendees.

Further interactions with and contributions to the other projects of the **SEURAT-1** Research Initiative include the following:

COSMOS esteblished the Cosmetics Inventory v1.0 as the first comprehensive compilation of cosmetics ingredients and related substances and is setting up a database of repeated dose toxicity and other toxicological information which will be shared with the **SEURAT-1** Research Initiative and also made publicly available. **COSMOS** is interacting with ToxBank for coordination of promotion and usage of the **COSMOS** database with the ToxBank Data Warehouse. A joint meeting of **COSMOS** and ToxBank delegates was held on 4 March 2013 in Lisbon, Portugal to discuss respective interactions.

Work package 4, working on physiologically-based pharmacokinetics (PBPK) and *in vitro* to *in vivo* extrapolation, has developed a first case study on multi-scale modelling with

acetaminophen, in single and repeated dose scenarios with data from DETECTIVE. Another line of work includes cooperation with NOTOX to predict adverse effects in the liver by coupling the internal metabolism (in the hepatocytes) with a simple 3D model allowing the simulation of the organ response in space and time. The model using an open source platform (KNIME/R) has been presented to the cluster.

These and other models and workflows will be made available to the other **SEURAT-1** projects, as well as, generally, support from **COSMOS** partner KNIME to build and distribute workflows developed or needed within the **SEURAT-1** projects.

4.5.7 Expected Progress within the Third Year

The **COSMOS** Project has a number of key goals with defined plans to achieve them. With regard to data collation, curation and sharing the long-term goal is to provide a database platform that will succeed **COSMOS**. In the third year of the project, the **COSMOS** database (DB) will be released. The **COSMOS** DB will include information on chemical structures and the **COSMOS** Cosmetics Inventory in addition to a data repository of toxicological and other information. It will have the ability to generate reports on the toxicity data associated with a chemical substance. The **COSMOS** DB will contain over 44,000 checked unique chemical structures, reference to the Tox21 and **COSMOS** inventories and legacy chemical assessment data (from US FDA PAFA). In total, more than 4,400 compounds have been included in the **COSMOS** cosmetics ingredients and materials inventory and these will be linked (where possible) to the CosIng database and SCCS reports. The **COSMOS** oral toxicity database will contain over 1,300 records for more than 1,000 chemicals including at least 750 cosmetics ingredients. In the medium term, research on the requirements and impact of a data governance framework for the **COSMOS** repository (the COSMOS space) will be provided and studies on toxicological data quality assessment will be published.

The third year of the **COSMOS** Project will provide the further extended TTC dataset, derived from the **COSMOS** DB. The TTC dataset will provide NO(A)EL values for approximately 700 compounds. There is a clear focus on the quality control of the TTC dataset with external experts contributing through ILSI Expert Groups. In addition to the analysis of the dataset to derive TTC values and advice, the TTC dataset will be transparent and made openly available to enable further development of the results and concepts as required. Related to the TTC analysis, a strategic decision framework for oral-to-dermal extrapolation will be developed. The aim of this framework is to identify chemicals for which a TTC value derived from the oral route may not be protective for dermal exposure.

Modelling will be a key activity in the third year of the **COSMOS** project. *In silico* modelling will be centred around developing, applying and utilising information from relevant Adverse Outcome Pathways. Specifically this will include the development of chemotypes for AOPs

relevant to organ level toxicity. Chemotypes extend and expand the structural alert concept by inclusion of other relevant physico-chemical properties. The software platforms developed in **COSMOS** are ideally suited to this purpose. The initial focus will continue with the study of the Liver X Receptor (LXR) with detailed consideration of how this receptor interaction can be described with molecular modelling approaches usually more associated with drug discovery.

A further aspect of the modelling in **COSMOS** that will be undertaken in the third year is the support and development of biokinetics models. Specifically, this will include the optimisation and calibration of PBPK models. Important here will be the use of QSAR models to predict parameters relevant to PBPK modelling i.e. log p, pK_a, fraction unbound, solubility etc. These will be supported by relevant information from *in vitro* assays to include metabolism and absorption. With regard to *in vitro* measurements of toxicity, extant data (e.g. for drug clearance) for the **SEURAT-1** standard reference compounds ('gold compounds') will be identified and strategies proposed for the better use and design of *in vitro* testing methodologies. A particular emphasis here will be the provision of advice to other **SEURAT-1** projects to develop their own biokinetics models supporting experimental designs in the field of repeated dose toxicity testing.

The KNIME workflow technology continues to support and provide an open access platform to present the results and models from the **COSMOS** Project. There will be continued 'behind the scenes' development of the technology to support KNIME for **COSMOS**. This will include improved database handling, development of new and bespoke nodes (functionalities), further implementation of the archiving functionality and increased web-portal usability. The end-end user will thus also be able to apply KNIME workflows through a web-browser-based version, without installing the software, and retrieve results immediately as a report in different formats.

4.5.8 Future Perspectives

Computational modelling is at the heart of the modern toxicological paradigm. The **COSMOS** Project within the **SEURAT-1** Research Initiative will provide the firm foundation required in this area to properly implement chemoinformatics to support risk assessment. Computational techniques will support toxicology in a number of key areas.

The **COSMOS database of toxicological information** will provide the backbone to the development of alternatives. **COSMOS** will provide an open database, both in terms of the structure and implementation but also the data contained. This will form a robust platform to collect, organise and mine *in vivo* and *in vitro* data beyond **SEURAT-1**. Therefore a strategic consideration must be to maintain the database ensuring it provides a facility to allow for more data storage. To support this activity the concepts of data (biological and chemical)

data quality assessment, as well as data governance, from **COSMOS** must be adopted and applied. The **COSMOS** database will also be linked to the ToxBank Data Warehouse.

COSMOS will develop Threshold of Toxicological Concern (TTC) approaches better suited to classes of cosmetics compounds. In order to progress the **COSMOS TTC models** into a possible SEURAT-2 Research Initiative there will be a strategic need to integrate mechanistic information. Specifically this should be led by implementing the Adverse Outcome Pathway (AOP) approach to provide the link from chemistry to toxicity pathways encompassing mechanisms. This will be at the basis of all approaches to tackle organ level toxicity. Specifically there will be a great strategic need to support *in silico* models, including TTC, by AOP considerations. The mechanistic considerations provide a cornerstone for the cross-cutting activities within the **SEURAT-1** Research Initiative and beyond.

COSMOS will provide a number of **innovative computational tools for toxicity prediction**. These will be built around the **COSMOS** database and Cosmetics Inventory. Of particular strategic importance beyond the **SEURAT-1** Research Initiative will be to develop categories from chemical knowledge derived from AOPs. These can be extended into more quantitative approaches to toxic potency, e.g. (quantitative) structure-activity relationships ((Q)SARs). Therefore the continued implementation of chemoinformatics tools, preferably freely available, will underpin strategic development of computational predictive toxicology.

Models for toxicodynamics and toxicokinetics will be developed within **COSMOS** and will form the foundation of research beyond **SEURAT-1**. It is already widely acknowledged that there is a great need to develop further the capabilities for *in vitro–in vivo* extrapolation. This will allow for the better application of results from cell-based assays to perform human safety assessment. Amongst the strategic requirements for SEURAT-2 will be kinetics modelling (e.g. through physiologically-based pharmacokinetic (PBPK) models); a better understanding of the effect of the properties of the test systems (e.g. sorption) and chemicals (e.g. volatility, stability) relating to extrapolation; and metabolism, its modelling and prediction.

Integrated efforts within **COSMOS** will also result in **workflows for toxicity prediction**. A finding from **COSMOS** will undoubtedly be that there is no simple computational method to predict organ level toxicity. Therefore, within SEURAT-2 there is a strategic requirement to develop and utilise open and transparent platforms, such as KNIME, to capture and implement modelling processes. Ultimately this will lead to a platform supporting data capture, storage and retrieval, links of chemistry to pathways through AOPs and open and flexible modelling for relevant endpoints to evaluate safety of chemicals to humans.

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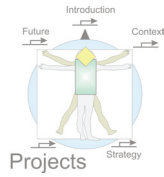
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4.6 NOTOX: Predicting Long-term Toxic Effects using Computer Models based on Systems Characterization of Organotypic Cultures

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4.6.1 Introduction and Objectives

Validated alternative assessment methods for long-term systemic toxicity are urgently required to cope with the complete ban (enforced 11 March 2013) on animal testing in Europe for cosmetic products. In the **NOTOX** initiative we have assembled experts for *in vitro* test systems together with scientists from the field of systems biology in order to establish new systems-based models for the prediction of long-term toxicity. **NOTOX** will develop and establish a spectrum of systems biology tools including experimental and computational methods for i) organotypic human cell and tissue cultures suitable for long-term toxicity testing with focus on the mode-of-action (MoA), and ii) the identification and analysis of adverse outcome pathways (AOP). The overall goal is to predict long-term toxicity (repeated dose) on the basis of these models and well-designed experiments using an iterative systems approach. Furthermore, predictive endpoints for repeated dose toxicity will be identified including molecular initiating events (MIE). The models will be multi-scale from molecular to cellular and tissue levels. Since testing on the target organism, human, is not possible, human organotypic cultures are applied to permit reproducible and transferrable testing of highest possible relevance. Multi-scale models shall eventually incorporate the obtained experimental data to predict human long-term toxicity. Ultimately it will be necessary to collect experimental data from all relevant tissues including the interactions between tissues and organs. Since liver plays a central role in metabolism, both concerning the inherent part as well as the part responsible for the xenobiotic conversion, we selected hepatic cultures for the **NOTOX** project. As human hepatic cells derived from stem cells are not yet readily available with sufficient functionality, for **NOTOX** we selected HepaRG, a hepatocarcinoma cell line and primary human hepatocytes (PHH). The HepaRG cell line has been shown to be closest to primary human cells in terms of metabolism of xenobiotics,

1- On behalf of all contributors

expressing important CYPs at high levels (*Kanebratt and Andersson, 2008a; 2008b*). For validation purposes and for the development of new techniques we also use the PHH. In these test systems viability and physiological toxicity-response parameters ('-omics') are monitored together with genetic, epigenetic and structural characterisation. Large-scale network models of regulatory and metabolic pathways and cellular systems, together with bioinformatics integration of human and across-species literature data, will lead to reliable toxicity prediction. The organotypic model systems are exposed to repeated low doses, in a long-term setup, of selected test compounds of industrial relevance with known mode-of-action (MoA) relevant to toxicity. These compounds are chosen from the gold compound list provided by ToxBank. The physiological effects of test compounds on the test systems will be monitored by determining '-omics' data (epigenomics, transcriptomics, proteomics, metabolomics, fluxomics) at various time points. Design of experiments will be assisted by toxicophysiology data curated from literature and databanks as well as from *in silico* simulations. As available, human target cells and organ-simulating devices from other projects (see previous project descriptions of *SCR&Tox* and *HeMiBio*) of the **SEURAT-1** Research Initiative will be implemented.

3D spatial organisation of tissue structures, cell-cell contacts and intracellular structural features will be characterised by 3D cryo-electron tomography and light/confocal microscopy. We will also use a newly established multi-scale mathematical modelling approach, where toxic effects on 3D organotypic cultures, including tissue microarchitecture as well as tissue function, can be simulated in a dose-dependent manner.

Effects upon long-term exposure to test compounds as monitored and measured by above-mentioned technologies will be analysed by bioinformatics methods. Data from databases, literature, experiments and simulation will be integrated through bioinformatics tools to create a knowledge base for quantitative understanding of adverse outcome pathways (AOP) and regulatory networks at the molecular level. These data will provide the basis for prediction models. Large-scale modelling of regulatory and metabolic pathways will simulate toxic responses starting from molecular initiating events (MIE). Since such large-scale computational systems biology models often comprise a large set of equations and include hundreds of thousands or even millions of data points, strategies will be developed using state-of-the-art multi-core and grid computing for analysis and exploration of these models.

The major objectives of **NOTOX** are:

- Supplying a versatile methodology for systems-based analysis and prediction of long-term toxicity of test compounds on organotypic 2D/3D cultures.
- Development and application of experimental and computational methods for continuous, non-invasive and comprehensive physiological monitoring (respiration, metabolomics, fluxomics, proteomics and peptidomics, epigenomics, transcriptomics, viability and toxicity reporters, cellular toxicity models) of organotypic test systems upon exposure to selected test compounds.

➠ Development and application of experimental and computational methods for the comprehensive characterisation of 2D and 3D organotypic cultures after long-term repeated dose exposure to selected test compounds (individual epigenetic chromosomal profiling, 3D electron tomography, 3D-topographic analysis and modelling, bioinformatics characterisation).

➠ Development of causal and predictive large-scale computer models based on the integration of the experimental data with available data (various databases) and high-performance grid computing for identification of predictive endpoints.

➠ Providing cheaper, more ethical, scientifically based testing strategies for repeated dose toxicity in order to meet the European legislative demands. For this purpose we will illustrate how computer models calibrated with *in vitro* experiments could be used in combination with human parameters to predict the possible toxicity in humans.

4.6.2 Main Achievements and Challenges in the Second Year

During the last year, extensive focus was on the **NOTOX** major outcomes, which are: (i) the development of 2D/3D culture systems; (ii) '-omics' analyses; (iii) systems level molecular model for description of compound effects in 2D/3D culture; (iv) mathematical multi-scale models for 3D organotypic cultures. The **NOTOX** knowledgebase is regularly updated with the data obtained in these four objectives. The analysed data will be published in peer-reviewed journals.

The main achievements in the last year are following:

➠ Establishment and maintenance of HepaRG 3D spheroid cultures for three weeks and characterisation by various functional tests, as well as immunohistochemistry for liver-specific expression of proteins and drug transporters. The objective of this case study was to assess the applicability of 3D HepaRG cultures in toxicity studies. These cultures were used to assess toxicity of acetaminophen and troglitazone. Inter-lab variability was also checked. The 3D HepaRG spheroid cultures are more sensitive to acetaminophen toxicity with an EC_{50} value similar to physiological *in vivo* concentration, than the 2D monolayer cultures. The results of this study were published recently (*Gunness et al. 2013*).

➠ Design and execution of a joint large-scale pilot experiment involving various **NOTOX** partners. The objective was to test acetaminophen acute toxicity using the '-omics' platform as a case study. All the relevant '-omics' analyses were carried out on the same batch of cells and within one experimental setup so that all '-omics' data are comparable. A model for acetaminophen acute toxicity has

been established. Two publications, describing results achieved in this study and the kinetic modelling of acetaminophen toxicity, are under preparation.

➠ Establishment of serum-free long-term (30 days) cultivation of HepaRG cells in monolayer cultures. A manuscript is submitted. The cultivation method will be transferred to organotypic 3D cultures (spheroid and sandwich cultures). This medium will be used in long-term repeated dose joint multi-omics experiments.

➠ Long-term repeated dose experiment with selected gold compounds for 28 days was carried out to obtain EC_{50} values. A manuscript on these results is under preparation. The results of this study are the basis for the choice of selected compound concentrations to be used in the long-term multi-omics experiment.

NOTOX is now dealing with challenges concerning the long-term testing, in 2D/3D, of selected drugs using '-omics'. Many small studies are underway to optimise the setup. Comprehensive bioinformatics analysis of multi-omics data is another challenge we are focusing on.

An additional focus is to get good structural microscopic data on the 3D spheroid cultures. The fixation protocol is optimised and preliminary electron microscopy data is very promising. Further microscopic characterisation (confocal microscopy) to obtain data for spatial temporal modelling is on-going. This will allow the development of physiological models characterising long-term toxicity of selected case studies with acetaminophen and valproic acid. Agent-based modelling of spheroid formation and activity as well as the effects of the selected compound, will be based on the obtained structural and activity data.

Based on the data obtained in the pilot experiment, further experiments with PHH are planned for IVIVE (*in vitro–in vivo* extrapolation). The setup for this work will be extensively discussed with modellers. This will provide an opportunity for extensive collaborations with COSMOS. The PBPK models generated in COSMOS will aid in the IVIVE.

4.6.3 3D Organotypic Cultures of HepaRG as a Tool for *in vitro* Toxicity Studies

Introduction

There is an urgent need for alternative *in vitro* human hepatic models, particularly those that retain their phenotypic characteristics for longer time periods, which can be used for more accurate assessment of drug-induced hepatotoxic effects in humans. The HepaRG cell line has been shown to retain liver-specific phenotypes, such as drug-metabolising activities, for several weeks after differentiation in 2D cultures. The HepaRG cultures have been chosen as the cell model for **NOTOX**. In this study, we wanted to determine if the HepaRG cells

can be cultured to a complex *in vitro* 3D model that reflects the *in vivo* 3D structure of a human liver. Therefore, the **NOTOX** project partners Saarland University and Karolinska Institutet established and characterised a 3D organotypic HepaRG model suitable for *in vitro* acute toxicity studies. The results showed that the 3D HepaRG organotypic cultures could be successfully obtained and the cultures were characterised in terms of liver-specific functionality and response to acute drug exposure (*Gunness et al., 2013*).

Approach

The 3D organotypic cultures of human HepaRG cells were obtained using InSphero's (InSphero AG, Zurich, Switzerland) high-throughput hanging drop technology. The 3D cultures were maintained for three weeks and assessed for: (i) liver-specific functions including cell metabolism, and phase I and III enzyme activities; (ii) expression of liver-specific proteins; and (iii) acute dose response to acetaminophen (acetyl-*p*-aminophenol, APAP), troglitazone and rosiglitazone.

Results

Morphology of 3D organotypic cultures: In *Figure 4.38*, the Hematoxylin and Eosin (H&E) staining of 20-day-old HepaRG spheroids distinctly shows viable cells with intact nuclei and cytoplasm throughout the spheroids. The presence of some fat droplets was observed within the hepatocytes, which may be due to aging, but a minimal amount of intrahepatic lipid storage is considered to be hepatoprotective (*Gibb and Anderson, 2008*).

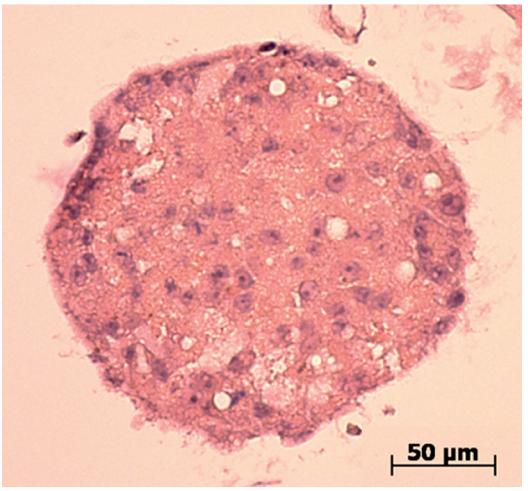

Figure 4.38 *Hematoxylin and Eosin (H&E) staining of a 3D HepaRG spheroid. Scale bar represents 50 µm.*

Liver-specific functions: Results indicate that the 3D cultures maintained liver-specific functions throughout the experimental period, as evidenced by the production of albumin and metabolites. The 3D cultures consistently produced higher levels of albumin, urea and glucose over the experimental period. Moreover, the levels of albumin and metabolites produced were always higher in the 3D versus the 2D cultures throughout the experimental period.

The expression of liver-specific proteins: Immunohistochemistry analyses on cultivation days 10 and 21 revealed that the 3D cultures expressed the liver-specific proteins albumin and CYP3A4, the most important drug-metabolising enzyme in the liver, throughout the culture period of three weeks (*Figure 4.39*). In contrast, the proliferation marker Ki-67 is only expressed at very low amounts, indicating an absence of cell growth and, therefore, stable spheroid diameters and cell numbers during the cultivation.

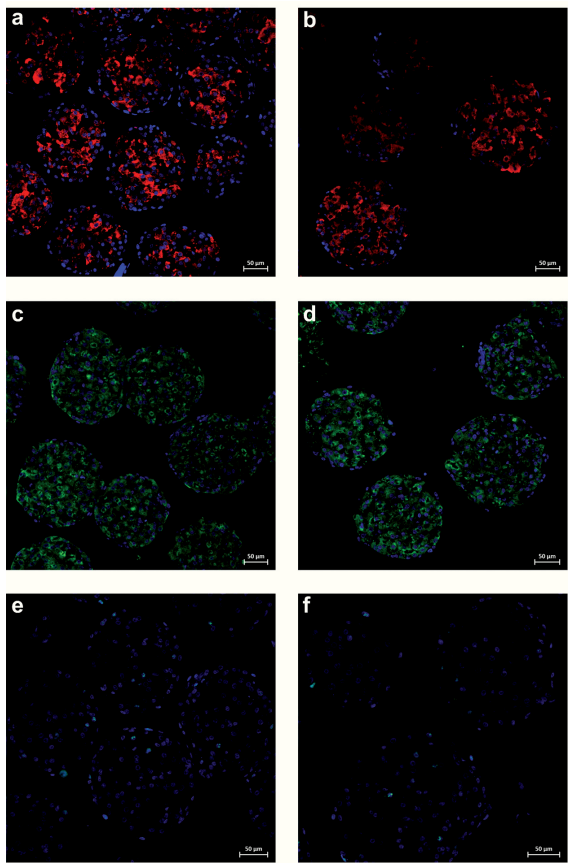

Figure 4.39 *Protein expression in 3D HepaRG cultures. The expression of CYP3A4 (a, b), albumin (c, d) and Ki67 (e, f) in 3D HepaRG cultures was assessed by immunohistochemistry. Scale bars represent 50 µm.*

CYP2E1 enzyme activity: The activity of the phase I drug metabolising enzyme CYP2E1 was assessed in the 3D cultures (*Figure 4.40*). The CYP2E1 enzyme is responsible for the metabolism of several xenobiotics of toxicological interest, including ethanol, carbon tetrachloride and the widely used analgesic and antipyretic drug, acetaminophen (*Knockaert et al., 2011*). The results illustrated that the CYP2E1 enzyme activity was consistently higher (approximately 11 fold) in the 3D versus the 2D cultures throughout the experimental period (*Figure 4.40*). The CYP2E1 activity decreased slightly from days 10 to 21, but nevertheless remained higher (at least 8 fold) in the 3D versus the 2D cultures.

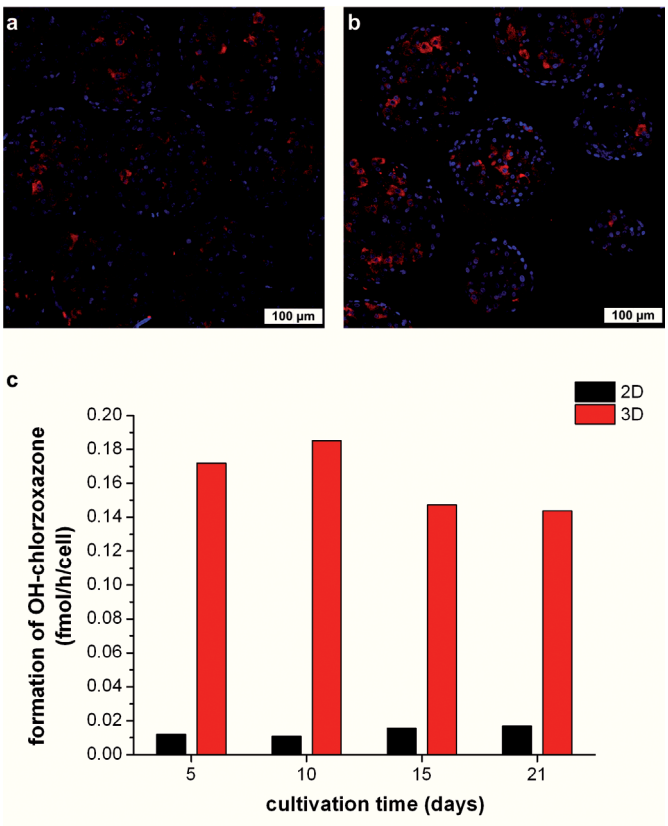

Figure 4.40 *a) CYP2E1 protein expression in 3D HepaRG cultures at day 10, b) CYP2E1 protein expression in 3D HepaRG cultures at day 21. Scale bars represent 100 µm. c) CYP2E1 enzyme activity in 2D and 3D.*

MRP2 transporter activity: The activity of the apical efflux drug transporter, multidrug resistance-associated protein 2 (MRP2), was assessed in 2D and 3D cultures. The MRP2 drug transporter, which plays an imperative role in the detoxification of xenobiotics (*Jedlitschky et*

al., 2006), is responsible for the active efflux of both endogenous and exogenous substances from cells, including bilirubin glucuronide, glutathione conjugates, cimetidine and methotrexate (*Ramboer et al., 2013*). The results clearly illustrate that both cultures possessed functional MRP2 drug transporter activity (*Figure 4.41*). However, the MRP2 functionality is located at the clusters of hepatocyte-like cells in the 2D cultures whereas we observed MRP2 activity throughout the whole 3D spheroids. The results also suggest that the cells within the spheroid cultures were polarised since MRP2 is expressed in the apical membrane of polarised hepatocytes (*Ramboer et al., 2013*). The MRP2 transporter activity results are supported by the immunohistochemistry results, which illustrated that MRP2 was expressed throughout the spheroids.

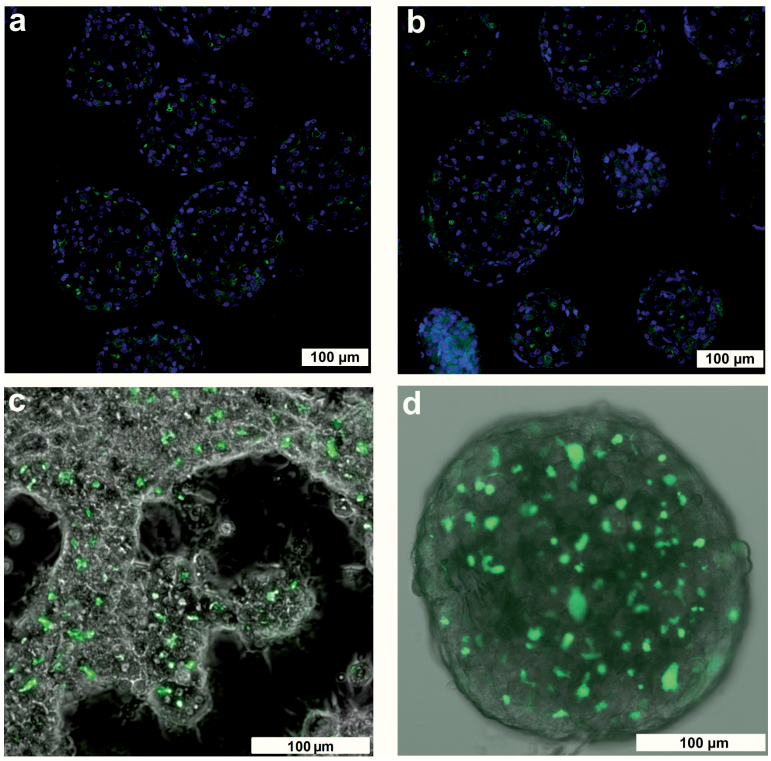

Figure 4.41 *MRP2 protein expression in 3D HepaRG cultures: a) at day 10, b) at day 21. MRP2 transporter activity in c) 2D and d) 3D HepaRG cultures. Scale bars represent 100 mm.*

Acute toxicity: The acute dose-response was assessed in the cultures following exposure to acetaminophen, troglitazone and rosiglitazone on days 5 and 21. *Table 4.4* provides a summary of the EC_{50} values.

Table 4.4 *EC_{50} values and corresponding ranges for acetaminophen, troglitazone, and rosiglitazone assessed on 2D and 3D HepaRG cultures upon 24-hour exposure on day 5 and day 22.*

		2D		3D		
Day		EC_{50}	range	EC_{50}	range	*p*-value*
5	Acetaminophen	26.3	24.9-27.7	2.7	0.6-10.7	< 0.001
	Troglitazone	40.9	26.7-62.7	398	334-474	< 0.001
	Rosiglitazone	195	181-211	122	107-138	< 0.001
22	Acetaminophen	34.6	30.6-39.2	10.1	5.3-19.3	< 0.001
	Troglitazone	301	294-309	> 500	n.d.	< 0.01
	Rosiglitazone	195	160-239	145	122-190	< 0.001

**p*-values indicate significant statistical differences between EC_{50} values of 2D and 3D cultures.

Compared to the 2D cultures, the 3D cultures were significantly more sensitive to acetaminophen exposure at both time points. The results are supported by those from the CYP2E1 enzyme activity assay where consistently higher enzyme activity was observed in the 3D versus the 2D cultures over the experimental period. The toxic metabolite of acetaminophen, N-acetyl-*p*-benzoquinone imine (NAPQI), is produced via metabolism by CYP2E1 (*McGill et al., 2011*).

In contrast to the 2D cultures, troglitazone was not toxic to the 3D cultures at both assessment time points. The mechanism of troglitazone-induced hepatotoxicity is not fully understood and it is uncertain whether troglitazone causes direct or idiosyncratic toxicity (*Isley 2003*). A plethora of mechanisms has been suggested including the formation of reactive metabolites, mitochondrial damage, apoptosis, inhibition of the bile salt export pump (Bsep) and activation of inflammatory responses (*Chojkier 2005*). The 3D cultures were more sensitive to rosiglitazone exposure at both exposure time points. Rosiglitazone is not hepatotoxic *in vivo*, however, rosiglitazone was found to be toxic *in vitro* in some donors of primary human hepatocytes (*Lloyd et al., 2002*).

Conclusions

Results from the study are promising and illustrated that the 3D HepaRG cultures were viable and maintained hepatocyte-specific function over the three-week cultivation period, including (i) production of albumin, urea and glucose and (ii) phase I, II and III enzyme activities. The cultures also expressed liver-specific proteins including albumin, CYP3A4, 2E1 and MRP2. Toxicity studies revealed that the cultures were more sensitive to acetaminophen and rosiglitazone, but less sensitive to troglitazone. Taken together, the results suggest that the 3D HepaRG cultures seem to be a more suitable model to study drug-induced hepatotoxicity.

4.6.4 Spatiotemporal Modelling

Introduction

NOTOX developed a strategy of crosstalk between experiments and modelling, which has already proven to be very efficient for several previous projects. The starting point is the experimental data provided by collaborators, which are usually supplied in the form of images. These images are analysed to extract quantitative information, both static parameters and parameters characterising the process, in case of spheroid formation. These parameters are used to run simulations, the results of which can be quantitatively compared with the experimental results. If the simulation results agree with the data, we can conclude that the model encompasses the primordial mechanisms for spheroid formation. If it is not the case, then our model has missed an aspect of the process, and it should be adjusted (e.g., by adding more complex mechanisms, as a long-range interaction between cells by the means of a chemo-attractant). In an iterative process, new simulations will be run until the model is able to fit the experimental data. When the model has gained sufficient confidence, hypotheses formulated in the model should be able to be validated experimentally.

Approach

We have developed a lattice-free mathematical model in which each cell is represented by a homogeneous isotropic elastic spherical object, moving subject to all forces on it and its own micro-motility. The cell-cell interaction forces have been modelled using an extended Hertz model (*Galle et al., 2005*), though we will also consider an experimentally validated interaction force model, the so-called 'Johnson-Kendall-Roberts model' (*Chu et al., 2005*) in the near future, as it can quantitatively change the results. The model parameters of our model are material and adhesion parameters determining the strength of physical forces, friction among cells and between cells and extracellular material, and the cells' micro-motility. Our first simulations are promising as they show that it is possible to calibrate physical parameters of the model in such a way that spheroid aggregation can be mimicked at least qualitatively.

We further continued to develop our software CellSys in cooperation with other funding (i.e., Virtual Liver Network). The architecture of the software permits a fast adaptation of the model to new biological questions and it has been extended to enable the crosstalk of the multi-cellular model to intracellular pathway models. The intracellular module in CellSys can now be linked to libraries of well-established pathway modelling (using SBML, **S**ystems **B**iology **M**arkup **L**anguage). Our software CellSys now permits us to simulate this entire process, from the cell seeding to the aggregation. Measuring and quantification in experiments is sometimes limited by either the available techniques or by the simple fact that measuring can cause interference. Mathematical models, alternatively, offer a unique chance to determine the influences of physical parameters (e.g. mechanical stress, strength of cell-cell and cell-

extracellular matrix (ECM) adhesion, cell motility, etc.) providing additional and necessary insights into the problem. Drug toxicity is a process involving several scales, from intracellular pathways to the entire organ. Thus only a multi-scale model will be able to grasp the complexity of the situation. CellSys is equipped with intracellular modules that enable us to incorporate quantification of biochemical pathways, linked to cell movement.

Results

The data that we collected from collaborators are bright field images of the aggregating cells at the bottom of the hanging drop, taken from underneath the drop, at several time points after seeding, and for different initially seeded cell population sizes. In the current model, we assumed that cells produce ECM proteins in large amounts, so that cells can migrate on/in this ECM. We set up our model by fixing parameters to match the experimental situation whenever possible (drop volume, cell size, etc.), and let the parameters vary within physically meaningful ranges. Through successive simulation runs, model parameters were optimised to bring the simulation as much as possible into agreement with the experimental results. We found that cells must be highly migratory to be able to form a round spheroid as quickly as shown in the experiments. But a large motility also leads to cell detachment from the spheroid, a phenomenon not observed *in vitro*. To avoid detachment of cells in the simulations, cell-cell adhesion also needs to be large. The parameter values that enable the best fit to the experimental data will be used as a basis to calibrate the future *in vivo* model where comparison with experimental data is less feasible.

We formerly used cellular automaton models to mimic the aggregation process. However, in cellular automaton models migration and interactions are rule-based, whereby the rules are expected to reflect physical laws correctly – yet this is not always the case. Moreover, cell migration, birth and death in spatial-temporal tissue organisation processes within cellular automaton models are defined on lattices and are usually inherently stochastic. Hence cell migration, growth and death processes which are entirely or mainly deterministic often cannot properly be captured by cellular automaton models. As a consequence we were not able to represent quantitatively the entire process of cell sinking and subsequent aggregation in the hanging drop method with cellular automaton models. In contrast to this, the lattice-free model type used in *Figure 4.42* is formulated in terms of physical laws and parameterised in terms of measurable parameters.

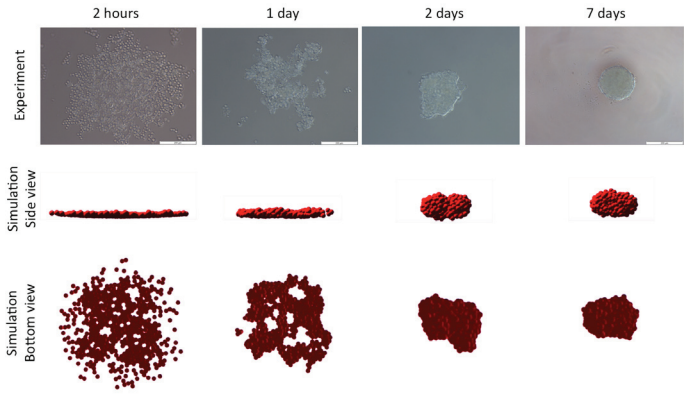

Figure 4.42 *Comparison of a spheroid aggregation of 500 cells in an in vitro hanging drop and in the simulation. Once cells are lying on the bottom of the drop, they progressively aggregate and finally form a round and compact spheroid after 4–7 days (top). With proper parameters, our model is able to reproduce the spheroid formation (bottom). However, an almost perfectly round spheroid could not be achieved during the same time span.*

Besides a general reorganisation of the code of CellSys to make it more modular and reusable, we implemented a coupling between the multi-cellular model and intracellular models, such as those developed by **NOTOX** partner Insilico Biotechnology. Drug toxicity involves both the metabolism of the drug at the intracellular level and the death of cells at the multi-cellular level. Until last year, CellSys comprised only the modelling of cells, without consideration of the processes that happen inside each cell. We have now extended the software to enable the modelling of both the cellular and intracellular parts, and, more importantly, the communication between the two. The intracellular model mainly belongs to two categories: logical models that represent each species with only two levels (present or absent), or models based on ordinary differential equations (ODEs) where each species has a concentration that can vary continuously over time, depending on the reaction taking place among the species present. As all parameters in the cellular model are continuous, we chose to focus on ODE models in which variables are also continuous.

A plethora of software supports the construction of ODE models. We took advantage of an existing library/API, SOSlib, and importing into CellSys an xml file describing SBML models, as well as the simulation of those models. SOSlib itself relies on the library libSBML to handle the objects representing SBML models and on the SUNDIALS library to simulate the ODEs. One ODE set is plugged into each cell, which will allow for the introduction of cell to cell variability in the future (*Figure 4.43*).

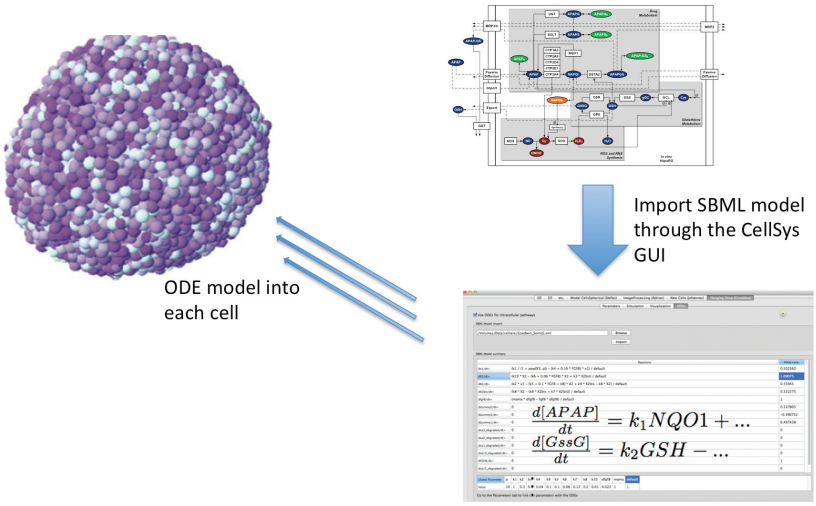

Figure 4.43 *Schematic of the integration of intracellular models (as ordinary differential equations, ODEs) in each cell of the population modelled in CellSys. Through the CellSys interface, the user can import an SBML model and couple it to the cell behaviour at the population level. The different colours of the cells denote different concentrations of an intracellular molecule.*

Conclusions

The modelling of different aggregation scenarios with a lattice-free model is, to the best of our knowledge, new and general because the hanging drop method is a standard method to form multi-cellular spheroids. Our innovation further consists in the link made between the ODE model and the cellular model: the user can define through the interface how the parameters from the cellular model should be linked to the intracellular concentrations. In the case of drug toxicity for example, the user can define that cells from the multi-cellular model die if the concentration of intracellular H_2O_2 produced during drug metabolism exceeds a given threshold, thus bridging the intracellular and population level.

4.6.5 Modelling of Ammonia Detoxification

Introduction

In the first phase of the **NOTOX** project we established two types of predictive computational models: (i) spatial-temporal models predicting liver toxicity and regeneration, e.g., in response to CCl_4 or acetaminophen (compounds of the ToxBank Gold Compound Selection); (ii) metabolic models of hepatocytes, including for example the ammonia metabolism. Therefore, it seemed feasible to integrate the metabolic model into the spatial-temporal model. The

integrated model simulates alterations in ammonia detoxification as a function of the degree of liver damage (*Heinzle et al., 2012*). The next question of interest was whether the model prediction was quantitatively correct.

Approach

We compared model predictions to experimental data of cultured human hepatocytes and existing data of CCl_4-exposed mice.

Results

The result showed a major discrepancy between model prediction and experiment. Interestingly, the ammonia concentration in the liver vein (representing the liver outflow) was much lower compared to the *in vivo* situation (*Figure 4.44*). A switch of the glutamate dehydrogenase (GDH) reaction from ammonia production to ammonia consumption has been reported previously for other cells (*Bonarius et al, 1998; Priesnitz et al., 2012*).

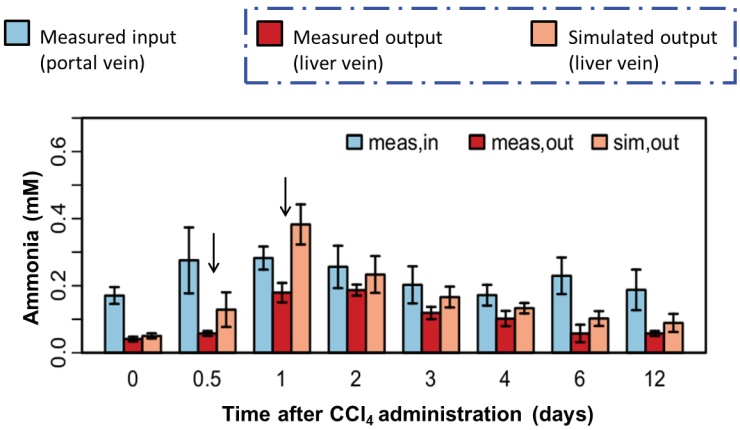

Figure 4.44 *The model prediction of ammonia concentrations is higher compared to the experimental data. The figure shows measured ammonia concentrations in the portal vein (liver inflow – blue) and the liver vein (liver outflow – red). The simulated ammonia concentrations in the liver vein are represented by the orange bars.*

Indeed, iterative cycles of experiments elucidated the following novel concept of ammonia metabolism (*Figure 4.45*). In normal periportal hepatocytes, GDH produces ammonia for further metabolism in the urea cycle. Upon liver damage and destruction of the pericentral compartment, ammonia leaks into the blood circulation leading to hyperammonemia

(*Figure 4.45*). However, GDH is also released from damaged hepatocytes. The released GDH catalyses an inverse reaction, which consumes ammonia to form glutamate. Thereby, damaged hepatocytes provide systemic protection against hyperammonemia. However, this detoxifying reverse GDH reaction can only take place until the a-ketoglutarate (a-KG) of the blood has been consumed by GDH. A therapeutic implication of this model prediction is that a-KG should be infused as soon as the endogenous a-KG decreases.

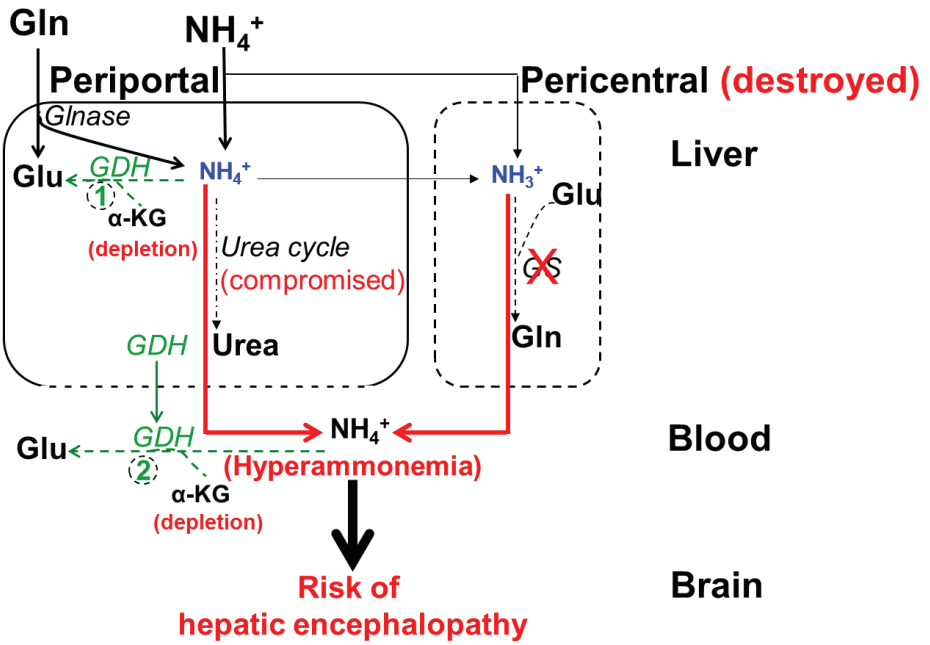

Figure 4.45 *Novel concept of ammonia detoxification obtained from iterative cycles of modelling and experiments.*

To test this prediction, hepatocytes sandwich culture *in vitro* systems were used (*Figure 4.46*). The *in vitro* system confirmed the switch of the direction of the GDH reaction; use of the GDH inhibitor pyridinedicarboxylic acid showed that GDH produces ammonia when the ammonia concentrations in the culture medium are low. However, at high concentrations ammonia is detoxified by GDH. When hepatocytes were exposed to high ammonia concentrations the GDH inhibitor clearly increased cytotoxicity.

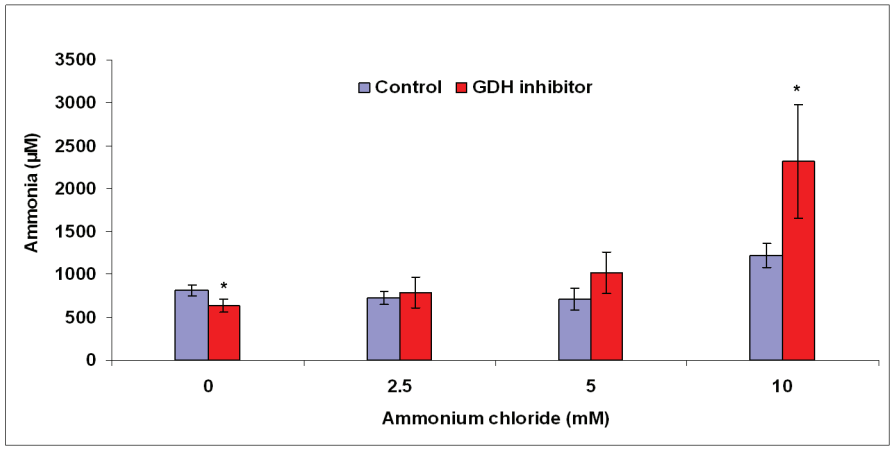

Figure 4.46 *Inhibition of GDH during exposure to high NH_4Cl levels shows increased ammonia concentrations in vitro. The figure shows measured ammonia concentrations in a culture medium of sandwich-cultured hepatocytes in absence (blue) and in presence (red) of GDH inhibitor.*

Conclusions

The model simulations predicted a so-far unrecognised mechanism of ammonia detoxification in primary human hepatocytes, namely a switch of the GDH reaction from ammonia production to ammonia consumption. *In vitro* experiments with hepatocyte sandwich cultures confirmed the model prediction. Further experiments are needed to study whether supplementation of a-KG indeed protects against hyperammonemia.

The results show that integrated spatial-temporal/metabolic models can indeed result in predictions that would not have been possible intuitively. Because of the novelty of the established models, we used substances of the 'gold compound selection' such as CCl_4 and acetaminophen for which data (including *in vivo* data) were already available. However, we expect that other compounds relevant for the cosmetic industry can be modelled in the future.

4.6.6 Modelling of Acute Toxicity

Introduction

In the second year of the **NOTOX** project, partner Insilico Biotechnology focused on the modelling of acute toxicity caused by overdoses of xenobiotics. Acetaminophen, which has also been recommended as a gold compound by ToxBank, was chosen as model substrate. Acetaminophen is considered as an essential drug by the WHO. However, overdoses lead to severe toxic side effects and even acute liver injury.

Approach

The original model presented in *Heinzle et al. (2012)* was modified to describe important processes related to relevant MoAs of acetaminophen. The steps to acute toxicity include (i) CYP-mediated reduction of acetaminophen to the metabolite N-acetyl-*p*-benzoquinone imine (NAPQI); (ii) depletion of cytosolic glutathione; (iii) binding of NAPQI to mitochondrial membrane; and (iv) excessive synthesis of reactive oxygen (ROS) and nitrogen species (RNS). The further progress of toxicity comprises the mitochondrial damage due to ROS and RNS and downstream events leading to cell necrosis and apoptosis.

Results

Our model for short-term toxicity comprised acetaminophen degradation, glutathione metabolism and ROS/RNS synthesis (*Figure 4.47*) for the prediction of ROS appearance as indicator of acute toxicity. The model has been pre-parameterised by literature values of kinetic parameters and compound concentrations, and first simulations have been performed to test model functionality. Thereby, **NOTOX** partner Insilico Biotechnology established the implementation of CYP-specific activity data from a large liver cohort (cooperation with Professor U.M. Zanger, Dr Margarete Fischer-Bosch Institute for Clinical Pharmacology, Stuttgart, Germany) into the cellular acetaminophen model and showed that cellular kinetic toxicity models could be a promising tool for fast and cost-effective analysis of population-wide drug response. The acetaminophen model verification was conducted on experimental time-series data generated in the **NOTOX** pilot experiment, where HepaRG cultures were exposed to two different acute-toxicity concentrations of acetaminophen and the time-dependent response was observed. The model described well the experimental data from the pilot experiment.

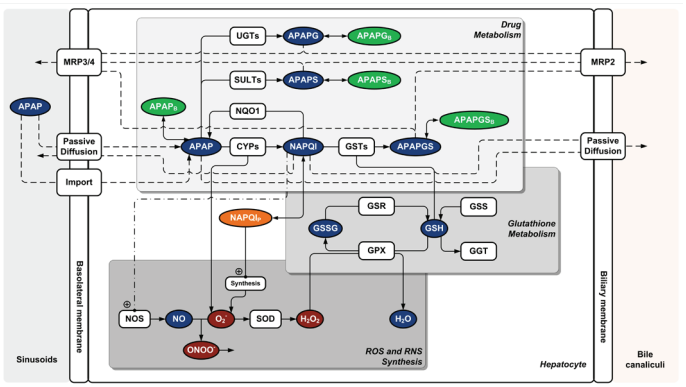

Figure 4.47 *Model of acetaminophen (APAP) metabolism, glutathione metabolism and ROS- and RNS-synthesis in hepatocytes. Acetaminophen is mainly degraded by phase II conjugation*

enzymes, UGTs and SULTs, and by phase I CYP-catalysed reduction to NAPQI. NAPQI is detoxified by GST-enzymes to APAPGS. Therefore, glutathione has to be regenerated from the amino acids glutamate, cysteine and glycine. NAPQI stimulates NO-synthesis and binds to the mitochondrial membrane, causing oxidative stress resulting in an elevated synthesis of reactive oxygen species O_2^- and H_2O_2, and reactive nitrogen species $ONOO^-$.

Currently, the HepaRG-central metabolism and its adaptation to acetaminophen treatment is analysed by **NOTOX** partner Saarland University. Further, complementary ^{13}C flux experiments and analyses are being conducted for estimating in more detail the contribution and importance of central metabolism to acetaminophen-induced toxicity mechanisms (e.g. to reveal glutathione turnover) in HepaRG cells.

Conclusion

In the modelling of acute toxicity, we successfully implemented *de novo* the combination of cellular drug metabolism, generation of reactive species and the link to the cellular scavenger system of glutathione. The chosen cell line, HepaRG, is a promising cellular system for toxicity testing since experimental data from the **NOTOX** acetaminophen case study experiment were successfully described by the acute toxicity model. Further, it was shown, that this model could potentially be used for fast, individualised or stratified toxicity screening in the future.

4.6.7 HepaRG Genome Sequence and Comparison of its Methylome with other Hepatic Cells

Introduction and Approach

To better understand genetic and epigenetic regulation we sequenced, for the first time, the genome of HepaRG differentiated cells on the HiSeq2000 (Illumina) platform. Reaching an averaged 45-fold coverage, we compared our results to the published HepaRG karyogram (*Gripon et al., 2002*).

Results

Genome of HepaRG: As illustrated in *Figure 4.48a*, the number of reads matching to chromosome 7 is significantly increased which is consistent with trisomy 7 already identified by Gripon and colleagues (*Figure 4.48b*). Secondly, we observed a heterozygous depletion at the proximal portion of chromosome 12, also consistent with the karyogram. In addition, SNP calling revealed 2,259,211 heterozygous variations, which were mapped and annotated. Our HepaRG genome map will be very valuable for the **NOTOX** consortium as well as other

SEURAT-1 projects using HepaRG cells, serving as a basis for transcriptomic and epigenomic analysis.

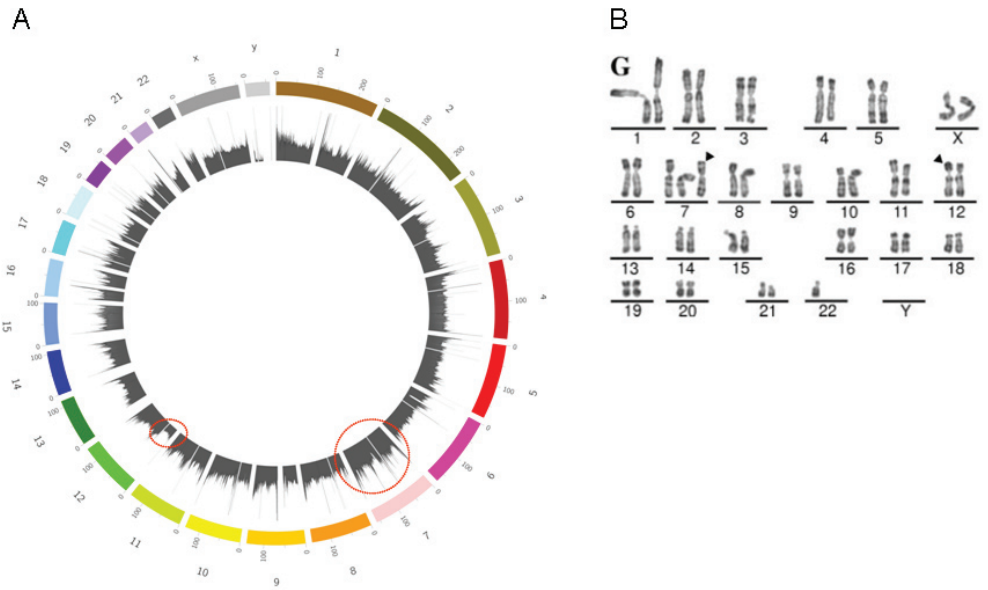

Figure 4.48 *a) Circular map of HepaRG genome showing the chromosomes and their respective read coverage. Trisomy 7 and heterozygous loss of the proximal chromosome 12 are highlighted with red circles; b) HepaRG karyogram taken from Gripon et al. (2002).*

Comparison of methylome of HepaRG, HepG2 and PHH: Preliminary analysis of gene expression and methylation data in HepaRG cells was carried out at Weizmann Institute, identifying clusters of coherent gene expression kinetics, and studying the potential regulatory circuitry underlying them (*Figure 4.49*). This coarse-grained description of the transcriptional network in HepaRG will serve as the basis for characterising more subtle responses during long-term exposure and low dose experiments.

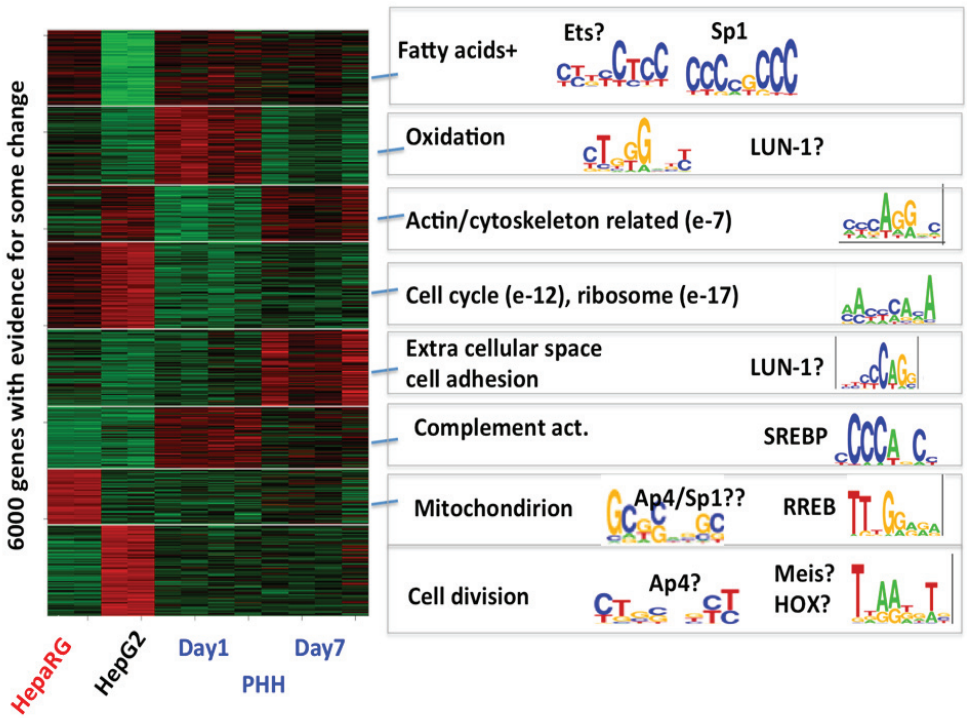

Figure 4.49 *Gene expression of HepaRG, HepG2 and exposed PHH cells was clustered using a variety of methods (K-means is shown here), as depicted by the heat map on the left (green – low; red – high). Encode-derived enhancer and promoter functional sequences were then associated with genes according to spatial proximity, generating a set of potentially regulatory sequences associated with each responding gene. Functional enrichment using GO annotation associated putative function with the gene in each cluster (Fisher exact p-value is shown). De-novo motif finding was performed using the inferno algorithm developed at Weizmann Institute, which is capable of fully controlling for background distribution heterogeneity in enhancer sequences. PWM logos of identified motifs are shown.*

4.6.8 Innovation

Spatial temporal modelling: CellSys, developed by a group of people within several projects, will be open-source software that can be used by various types of users (biochemists, biologists, engineers, etc.). The interface is user friendly. Model parameters obtained by experiments can be readily used as starting parameters for *in vivo* simulations with this model. Behind the scenes, though, lies a truly multi-scale spatiotemporal model on the population and intracellular levels (*Figure 4.50*).

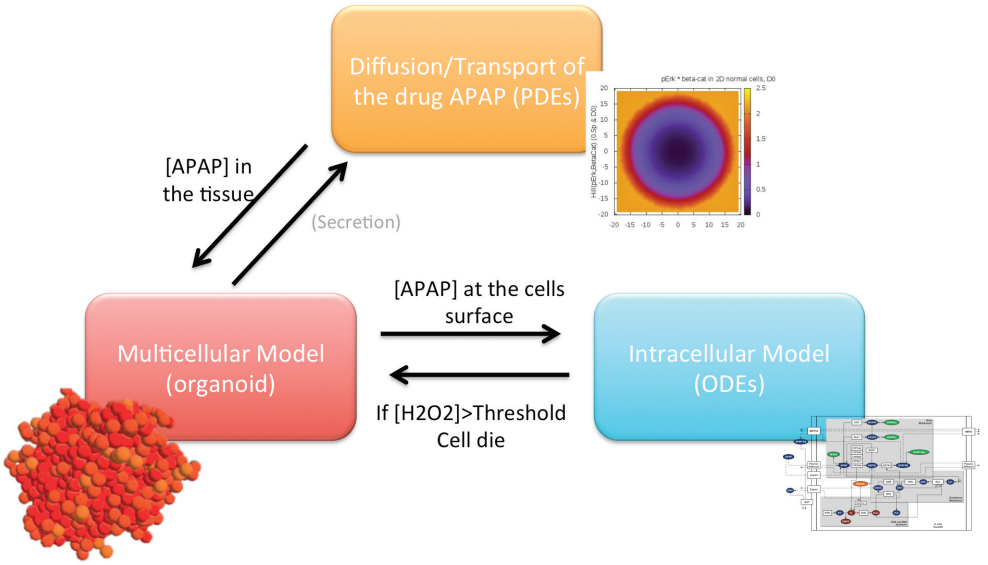

Figure 4.50 *Communication between the three model scales (extracellular, cellular and intracellular). In the near future* ***NOTOX*** *will further extend the software CellSys so that it also incorporates a solver for PDEs. The drugs can diffuse in the medium, be detected at the cell surface and trigger an intracellular response, which in turn will impact the cell behaviour.*

Large multi-scale long-term toxicity models: As described previously, multi-scale models cover complexity at different levels (*Niklas et al., 2013*). By extending metabolic models with signalling and regulation networks, these multi-scale models would be able to reflect long-term repeated doses and describe gradual manifestation of toxicity. The development of methods for *de novo* coupling of signalling, regulation and metabolism networks by **NOTOX** partners Insilico Biotechnology, Cambridge Cell Networks and Weizmann Institute will be an important step towards long-term toxicity modelling. This approach will further enable the consideration of gene regulation, epigenetic changes and individual specificity, influencing transcriptomic alterations and varying metabolic capacities or drug toxicity sensibilities.

4.6.9 Cross-Cluster Cooperation

NOTOX partner Insilico Biotechnology cooperates closely with partners from the **SEURAT-1** project COSMOS, both in-house as well as with other groups (Joint Research Centre in Ispra, Italy, and Institut National de l'Environnement Industriel et des Risques) focusing on the combination of cellular network models with structured organ models and PBPK models for simulation of drug distribution in the whole body. This concept was presented recently (*Diaz et al, 2013*).

Saarland University contributed to the joint workshop organised by the **SEURAT-1** project *HeMiBio* with a lecture presenting **NOTOX** activities in the field of bioreactors and engineering of cells (Gent, Belgium, 16 June, 2012; see also report in chapter 4.10.9). Furthermore, members from the **NOTOX** consortium participated in workshops organised by the Mode-of-Action Working group and the Safety Assessment Working Group in Tübingen, Germany (13-14 November, 2012; see also workshop reports in chapter 4.10.5.4 and chapter 4.10.8.2, respectively). These events were organised by COACH.

Other collaborative efforts for biokinetics are continuing with the **SEURAT-1** project ToxBank, and especially with the Mario Negri Institute for bioavailability studies.

In addition, we attended the 3rd annual **SEURAT-1**-meeting in Lisbon and participated in the workshop on data integration by ToxBank, which will be an important aspect in data management, exchange and implementation into models. Intense discussion also took place with partners from the **SEURAT-1** projects *HeMiBio* and COSMOS in the market place sessions during the meeting (see chapter 4.9.2).

4.6.10 Expected Progress within the Third Year

Large scale multi-omics experiment on acute acetaminophen toxicity: Joint modelling and bioinformatics analysis will be completed and results of acetaminophen case study will be published.

Long-term repeated dose case study using valproic acid: A joint multi-omics experiment is planned using valproic acid in a long-term repeated dose toxicity study. Major focus will be on steatosis as the mechanism of valproic acid toxicity. The experimental design is carried out jointly by both experimentalists and the modellers. Complementary long-term experiments, such as using 3D spheroids (Saarland University and Karolinska Institutet) and sandwich cultures (Leibniz Research Centre for Working Environment and Human Factors) to support modelling are also planned for valproic acid with HepaRG cells and PHH. In parallel the models for acute toxicity will be extended to allow for the simulation of repeated doses. The NOTOX partners will further construct signalling and regulation pathways that are induced by drug exposure and linked with central and drug metabolic systems. A special emphasis will be on epigenetic analysis performed by Saarland University and comprehensive bioinformatic data analysis carried out by Weizmann Institute. This will also lead to a better understanding of adverse outcome pathways.

^{13}C metabolic flux analysis: Saarland University is currently working on ^{13}C metabolic flux analysis for estimating the metabolic fluxes within the central carbon metabolism of the

HepaRG cells. Using these methods the effects of drugs on the cellular metabolism in acute and chronic toxicity studies will be investigated in detail.

Proteomic characterisation of HepaRG cells: Proteomic data for HepaRG is still not available or scarce. In cooperation with Biopredic International, Cambridge Cell Networks has now identified 1800 proteins using a combination of SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and tandem mass spectrometry (GeLC-MS/MS). As well as main metabolic pathways such as glycolysis and TCA-cycle, pathways also predominately active in liver (e.g., urea cycle, fatty acid synthesis, glycogen metabolism and detoxification of xenobiotics) were covered. The next steps will include an investigation into batch to batch variations of HepaRG and comparison to primary human hepatocytes, the current 'gold standard' for *in vitro* drug testing.

The proteome characterisation of the acetaminophen case study, where 1100 proteins have already been quantified, will be completed. The results so far are consistent with the current knowledge of acetaminophen metabolism, i.e., that the drug is mainly detoxified via glucuronidation rather than sulfation. Further studies will comprise quantification of additional phase I and II enzymes involved in acetaminophen metabolism using selected reaction monitoring (SRM). Extensive proteomics analysis of the planned valproic acid case study will be carried out in the coming year.

NOTOX Knowledgebase: Cambridge Cell Networks will continue systematic knowledge extraction, such as text mining and expert curation of the relevant scientific literature particularly related to aforementioned case studies, and exploring further case studies. The **NOTOX** Knowledgebase with information annotated from the public domain (scientific literature and existing knowledge) related to long-term toxicity will be used for the integrated analysis of multi-omics data for acetaminophen and valproic acid.

Spatiotemporal modelling: Model re-organisation scenarios of the multi-cellular spheroids when exposed to drugs, based on electron microscopy and light microscopic data, will be investigated. For this purpose, the intracellular ODE-models developed by Insilico Biotechnology will be coupled to the multi-cellular framework presented above. This includes linking the intracellular model to the diffusion of species/ligands, and a proper diffusion modelling of molecules inside the spheroid. Necessary data particularly on spheroids will be supplied by partners Netherlands Cancer Institute, Leibniz Research Centre for Working Environment and Human Factors, and Saarland University.

IVIVE: The major role in IVIVE will be with modelling partners National Institute of Research in Informatics and Automation, and Insilico Biotechnology, to link cellular models to

corresponding cell tissue and organoid models, but also with experimentalists for the setting up of corresponding validation experiments on 2D/3D cultures. In the third year, Insilico Biotechnology will focus on the development of appropriate model validation approaches, which will be necessary for the extrapolation of toxicity from *in vitro* HepaRG to PHH and to *in vivo*. This will be based on the mode-of-action.

3D cryo-electron tomography: Structural characterisation in **NOTOX** is performed using high-resolution 3D cryo-electron tomography (3D cryoET) for sub-cellular macromolecular objects, and serial section-scanning electron microscopy and standard electron microscopy for spheroid-level information. The techniques are still in development with the ultimate goal of detecting early micro-tissue, cellular and sub-cellular structural changes imposed by the application of test compounds. In 3D cryoET the focus was on improving the contrast, resolution and throughput. We have chosen the ribosome, a suitable macromolecular target, to be used as a test for identifying the induced structural changes. One dataset depicted in *Figure 4.51* is a reconstructed vitreous section of *Mycobacterium marinum* treated with hygromicin B. The key message is that even under the difficult conditions in all tested datasets major cellular features, such as membranes, putative ribosomes and other structures of interest can be detected with very good contrast and clarity that far surpasses current mainstream techniques such as weighted back-projection.

Figure 4.51 *3D cryoET reconstruction of the Mycobacterium marinum dataset. One slice from the density is shown on the left; zoomed-in detail on the right. We achieve high contrast with good clarity of the structures of interest.*

This sets a solid foundation for continuing with the next steps on the project roadmap, specifically: designing algorithms and software for analysis of the obtained volumetric reconstructions, and linking the structural data to toxicologically relevant processes and molecular initiating events (MIEs). On the spheroid level we have shown using standard EM that spheroids of proper size contain a large fraction of viable hepatocytes. These will now be investigated in 3D to, for example, elucidate 3D structures of bile canaliculi.

4.6.11 Future Perspectives

We see a bright future for systems oriented methods in toxicology. A broad '-omics'-based analysis will very likely detect even sub-toxic deviations from a reference state. '-omics' methods, particularly epigenomics, are expected to develop tremendously and will provide invaluable information for predictive toxicology. Metabolic flux analysis combined with sensitive metabolome analysis will be more easily applicable with further developed techniques for modelling and parameter estimation. This is particularly important since new compound targets and mechanisms are usually unknown. A systems biology approach involving multi-scale predictive models will also allow prediction of whole organism effects, particularly systemic effects, with increased reliability.

In the **NOTOX** project, eventually easily applicable methods of analysis will be developed so that they can be readily transferred to other cellular systems, for example,e those being developed or optimised in other projects of the **SEURAT-1** Research Initiative. *In vitro* test systems are of utmost importance for toxicity assessments without the involvement of animals. In **NOTOX** we have already made significant progress in the establishment of long-term 3D organotypic cultivation techniques, which are considered a major part of long-term toxicity assessment systems. The ultimate goal is to create cellular systems that are as simple as possible, for example using sandwich culture, or spheroid cultivation utilising new techniques that provide a high degree of reproducibility and predictive power. Miniaturised cultures, such as single spheroids or even functional organoids, that are presently limited in their applicability due to the lack of sufficiently sensitive analytical techniques, will also gain increasing relevance for a systems-wide characterisation.

Multi-scale mathematical and bioinformatic computer models will describe the mode-of-action from molecular to tissue to organism levels, thus improving the predictive power. For a systems biology approach, this will provide an excellent starting point for further refining strategies for obtaining improved prediction using a well-balanced combination of experimental and modelling techniques.

A further step in the upcoming years is the enhancement of extraction and analysis algorithms that will enable robust characterisation of the adverse outcome pathways (AOP) already in *in vitro* culture systems. Ultimately in the future, the goal is a routine assessment and semi-automatic reasoning about general compounds and modes-of-action. This will require the study of significantly smaller complexes and more subtle structural changes, to recognise the adverse effects as early as possible. Finally, the multi-scale models should allow *in vivo* extrapolation of long-term toxicity prediction in humans (IVIVE), which will be a great advance in the direction of alternatives to animal testing.

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Awards and Prizes

Daniel Müller and Sebastian Klein received bursaries for young scientists for the Systems Biology of Liver conference held in Luxembourg 21–23 February 2013.

A figure from a recent publication (*Gunness et al., 2013*) was used on the cover of *Toxicological Sciences* (Vol. 133, Is. 1).

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4.7 ToxBank: Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology

Barry Hardy[1]

4.7.1 Introduction and Objectives

ToxBank is the cross-cluster infrastructure project whose activities support the collaborative research activities of all **SEURAT-1** partners and consortia. To that end **ToxBank** has established a dedicated web-based warehouse for toxicity data management and modelling, a 'gold compound' database and repository of selected test compounds to use across the cluster to support the mode-of-action (MoA) framework, a physical compounds repository, and a reference resource for cells, cell lines and tissues of relevance for *in vitro* systemic toxicity research carried out across the **SEURAT-1** Research Initiative.

The primary objectives of **ToxBank** are to:

- collaboratively establish the requirements for data management and modelling, chemical compounds, and cell and tissue biological reagents for systemic toxicity research methods across all projects of the **SEURAT-1** Research Initiative;
- establish a data warehouse of linked resources which house and provide access to a centralised compilation of all data from the **SEURAT-1** Research Initiative (both experimental and processed data), public data from high-quality repeated-dose *in vivo* and *in vitro* studies, together with ontologies and computer models generated from the data;
- develop web-based interfaces for linking and loading raw and processed data into the data warehouse infrastructure as well as accessing the data and modelling results, including methods for searching, visualisation, property calculation and data mining;

1 - On Behalf of the ToxBank consortium

➠ specify standardised requirements for annotation and submission of '-omics' and functional data produced by the projects of the **SEURAT-1** Research Initiative to the data warehouse;

➠ design and implement a standards-based interoperable system enabling the integration of tools and distributed resources from multiple sources including project partners of the **SEURAT-1** Research Initiative and other projects (e.g., FP6, FP7, IMI, ToxCast, etc.);

➠ select 'gold standard' test compounds ('Gold Compounds') having high-quality data and providing chemical and biological diversity across a range of modes-of-action (MoAs) for repeated-dose toxicity endpoints;

➠ create an information resource and database for the import, curation, acceptance and storage of quality data related to the Gold Compounds;

➠ support education and ensure compliance within the **SEURAT-1** Research Initiative with procedures and data submission requirements and obligations to fulfil an integrated data analysis strategy across the complete **SEURAT-1** programme;

➠ establish a physical repository of test chemicals used within the projects of the **SEURAT-1** Research Initiative, characterising relevant physicochemical properties including stability, purity, isomeric form and binding properties, and standardised sample handling and operating procedures;

➠ establish criteria and procedures for the delivery of high-quality and acceptable sources of antibodies, cell and tissue materials for toxicology testing and control;

➠ establish a network of key suppliers of biological materials operating under consensus standards for quality that address the program research needs and anticipate future validation and regulatory issues;

➠ establish user community (research and industry) requirements for reference materials, assays and biomarkers;

➠ develop the capacity for increased adoption and use of standards for data, experimental procedures (protocols, SOPs), and best practices for analysis;

➠ develop cluster capacity for establishing quality and reliability goals in methods;

- develop cluster capacity for the reliable estimation of uncertainty in predictive models;

- establish a sustainable infrastructure of resources supporting and servicing all current requirements for systemic toxicology R&D that is extensible to future requirements for validation and risk assessment acceptance for industrial and regulatory needs.

4.7.2 Main Achievements and Challenges in the Second Year

The **ToxBank** consortium made considerable progress during 2012 towards achieving the **ToxBank** Data Warehouse objectives, which is described as the selected highlight in the following section 4.7.3.

The main objective of the **ToxBank** Compound Database within the first reporting period was to establish selection criteria for **ToxBank** Gold Compounds, to establish a database for Gold Compounds and to import an initial set of compounds into the database. For the selection of Gold Compounds the Gold Compound Working Group (GCWG) was formed. The GCWG selected candidates for cross-cluster testing based on chemical space, promiscuity and MoA. Selection criteria and standard operating procedures for data quality control, acceptance, processing and analyses of **ToxBank** Gold Compounds have been published (*Benfenati et al., 2012*). A collaborative **ToxBank** Gold Compound information wiki was established and populated with an initial set of 23 reference compounds for the cluster, including information about chemical identities, adverse effects, toxicity mechanisms and therapeutic targets (www.toxbank.net).

A physical repository for test chemicals to be used within **SEURAT-1** has been established in Italy at the **ToxBank** partner site. It addresses the characterisation of the chemical structure and relevant physicochemical properties of the test chemicals used within the cluster projects both through data collection, software predictions and experimental analysis. The Gold Compounds have been characterised with physicochemical properties in relation to their adoption for testing; the evaluation was concentrated particularly on structure and isomeric form, purity, stability, solubility, binding properties and volatility. To ensure a higher standardisation through the use of the same product within the **SEURAT-1** Research Initiative, specific recommendations on products and sources for the chemical standards have been provided on the basis of the evaluation of the highest purity available, the reliability of information and traceability, and suitability for cell culture application.

A framework for a **ToxBank** Biomaterials wiki has been developed, and populated with information on cells (stem cells and primary cells) and reagents (antibodies, growth factors, etc.) and suppliers. The completed wiki will contain general information on biological materials

and suppliers, and will also provide a discussion forum for use by investigators across the **SEURAT-1** Research Initiative. A summary of existing European biobanks and repositories has been compiled. An evaluation process has been created based on feedback from suppliers, as an indication of how they meet the quality, research and regulatory criteria established for these materials. In addition a directory of suppliers of cells has been initiated to include information on the minimal data sets (quality control, safety testing, and characteristics) as well as compliance with established best practice.

The **ToxBank** project has established initial critical infrastructure and service functions to support all **SEURAT-1** research projects providing a centralised and standardised set of data resources, compounds, and biological samples accompanied by protocols, SOPs and guidance. The provision of quality sources of compounds, cells and tissues for research will promote novel human cell-based assays that will facilitate more accurate evaluation of toxicity by the cluster. These resources will help ensure that the alternative *in vitro* assays developed by research activities on the **SEURAT-1** Research Initiative are guided and supported from an early stage of design, to maximise their potential of reaching the pre-validation stage as defined by ECVAM, and eventual validation and regulatory acceptance such as required under REACH or the Cosmetics Directive.

4.7.3 Selected Highlight: The ToxBank Data Warehouse

Introduction

The **ToxBank** Data Warehouse provides a web-accessible shared repository of know-how and experimental results supporting **SEURAT-1** research objectives such as toxicity pathway elucidation and biomarker discovery. Information within **ToxBank** is uploaded from the research activities of the **SEURAT-1** partners as well as from relevant data and protocols from other sources, such as public databases containing information on the standard reference compounds ('Gold Compounds'). The data is collected to enable a cross-cluster integrated data analysis leading to the prediction of repeated dose toxicity. Core technologies used in the development of the data warehouse include the use of the ISA-tab universal data exchange format, REpresentational State Transfer (REST) web services, the W3C Resource Description Framework (RDF) and the significant reuse of the recently developed OpenTox standards. We describe here the design and development of the **ToxBank** Data Warehouse based on requirements of the **SEURAT-1** Research Initiative and the implementation based on open standards.

State of the Art

The primary goal of the **SEURAT-1** Research Initiative is the development of human safety assessment strategies which may be used to replace (replace component of '3Rs' principle)

repeated-dose systemic toxicity testing historically carried out in animals. To achieve the ambitious overall goals of the **SEURAT-1** Research Initiative, it will be necessary to develop surrogate *in vitro* and *in silico* methods that are predictive of human toxicity. A chain of associations from human toxicity to animal models to *in vitro* models to *in silico* models is required, validated in the context of compounds with well-described toxicity-relevant properties that are thoroughly characterised at each node in the association chain. Such compounds comprise a set of 'Gold Compounds' around which all cluster-level efforts will be focused (the selection procedure was extensively described in the second **SEURAT-1** Annual Report (*Benfenati et al., 2012*) and the compound list is given in section 4.10.3.3). This will ensure the development of robust methods, based on high-quality data, with well defined applicability domains and the potential to extend research developments into validation exercises for larger compound sets and across multiple laboratories.

The origin of our **ToxBank** consortium and the planned approaches stemmed from a number of initiatives and interactions. Partners had coordination, requirements and technical leadership roles on the FP7 OpenTox (www.opentox.org) project (*Hardy et al., 2010*), which established an integrating computing framework for predictive toxicity data management, modelling, validation and reporting satisfying research and REACH regulatory requirements. The OpenTox framework has been implemented with public open standards and interoperability goals, which has been leveraged strongly in an extensible way to the creation of the **ToxBank** data management and analysis infrastructure. In addition to Open Source components, OpenTox supports the integration of both commercial and academic resources through its published application programming interfaces (APIs), which can support applications and workflows developed by solution providers.

Approach

In developing infrastructure such as the data warehouse, **ToxBank** is taking advantage of existing open standards, particularly the OpenTox project (*Hardy et al., 2010; 2012; Jeliazkove & Jeliazkov, 2011; Tscheremanskaia et al., 2012; Willighagen et al., 2011*). OpenTox developed a standard framework for interoperable predictive toxicology support (www.opentox.org/). It makes extensive use of REpresentational State Transfer (REST)-based web services (*Jeliazkova & Jeliazkov, 2011*) for interaction with different geographically distributed services necessary to support predictive toxicology data management, algorithms, modeling, validation, and reporting. Extensions were made to the OpenTox framework to support additional activities needing support by **ToxBank** within the **SEURAT-1** Research Initiative.

ToxBank uses the Investigation/Study/Assay (ISA) infrastructure open source desktop software suite (*Sansone et al., 2012*). Ontologies and a domain-specific **ToxBank** keyword hierarchy are used to enrich datasets by adding enough experimental metadata to make the

archives comprehensible and reusable. The ISA2RDF tool of the ISA-tab framework facilitates conversion of investigation meta-data into the semantic web standard Resource Description Framework (RDF) format.

The data in the **ToxBank** Data Warehouse is being collected to enable a cross-cluster integrated data analysis leading to the prediction of repeated dose toxicity within an MoA framework, based on a detailed understanding of the technologies, requirements and work practices developed across the cluster. Semantic web technologies are likely to be useful for integration of internal information from **SEURAT-1** with external information from database resources around the world (*Wild et al., 2012*).

The **ToxBank** consortium has made considerable progress towards the **ToxBank** Data Warehouse objectives. Specifically, based on an extensive requirement gathering from all **SEURAT-1** consortia and an analysis of this data, a preliminary version of the **ToxBank** Data Warehouse has been implemented that will provide access to all experimental, raw and processed data and protocols alongside relevant public information. This includes the development and/or customisation of web-based interfaces for linking and uploading data, including raw, processed and model results. All steps of any experiments will be linked to protocols describing the procedures. A web-based Graphical User Interface (GUI) for searching, browsing, and filtering the results has been implemented to provide access to all protocols and data across the cluster in a way that is sensitive of any intellectual property restrictions on access. The system has been implemented as a series of REpresentational State Transfer (REST)-based web services which will enable interoperability with other systems across the **SEURAT-1** Research Initiative as well as external resources.

Results

Based on an extensive requirement gathering from all **SEURAT-1** consortia and an analysis of this data, a first version of the **ToxBank** Data Warehouse has been implemented that will provide access to all experimental, processed data and protocols alongside relevant public information. We describe here its design and development.

Approach to collecting requirements: To ensure the web-based **ToxBank** Data Warehouse meets the needs of the individual projects and program, user requirements were gathered from partner participants in all ***SEURAT-1*** consortia. The **ToxBank** consortium used a methodology referred to as contextual inquiry/design for the collection of information to use in developing the system requirements (*Holtzblatt et al., 2005*). Interviews were conducted with individual **SEURAT-1** scientists covering a variety of tasks including the development of human safety assessment strategies, integrated data analysis, mining and modelling, the use and documentation of differentiated stem cell and organ-specific cells, the use of cell

culture systems, stem cell differentiation protocol development, the use of '-omics'-based biomarker assays and assay data, assay development based on 3D architectures, bioreactors and miniaturised simulated organ substructures, the use of functional parameters of cellular toxicity, the use and optimisation of (Q)SAR, read-across and grouping methods, and the use of pathway identification systems.

Shortly after each interview there was a debriefing session with other **ToxBank** team members. All the tasks or sequences recorded throughout all the interviews were then consolidated where common tasks were performed by different scientists. These consolidated sequence models served as use cases summarising the work across the **SEURAT-1** Research Initiative. A second consolidation used the notes recorded from the interview to create an *affinity diagram* (a hierarchical view of all notes collected). The affinity diagram and the sequences (including any consolidated sequences) were used as the starting point for the system design.

Analysis of requirements data: An analysis of the data resulted in five top level categories:

➠ The creation, use and sharing of procedures. This is the most populated branch based on the number of individual notes, reflecting its importance in the current **SEURAT-1** activities. An important aspect of this work is how the different consortia manage these protocols, which include placing restrictions on their use, gaining access to other protocols, sharing protocols, using protocols, and distributing protocols.

➠ Accessing and generating data. Data is required across the entire **SEURAT-1** Research Initiative and is also being generated in most of the activities. Primarily local solutions are currently being adopted for storing the data. The use of Excel is pervasive across the cluster; however, there is a clear need for developing improved approaches to integrate different sources of information together.

➠ Processing and analysing data. In many situations the data is collected and then further processed. Today, this analysis is challenging as a result of the difficulty in accessing the data which requires considerable manipulation and use of local statistical analysis tools.

➠ Issues that affect work. There are many additional factors (other than scientific considerations) that influence the activities of the **SEURAT-1** Research Initiative. These include intellectual property, legal, regulatory, the need to publish, and lack of resource issues. A cluster level set of terms and conditions for controlled sharing and use of data has been developed in parallel to the warehouse development.

➠ Assessing toxicity. This is one of the smallest branches in the affinity diagram, in terms of number of notes, concerns how these technologies were used to assess toxicity, reflecting the current cluster activities of the **SEURAT-1** Research Initiative.

A number of critical issues and ideas were drawn out from an analysis for the requirements data. The need to develop a solution to manage, register, assign a status, comment, peer-review, and sensitively share the diverse protocols being generated throughout the cluster was seen as a high priority. Handling data presented a number of complex problems as a result of the diversity of the experiments being employed as well as the different workflows currently being adopted across the **SEURAT-1** Research Initiative. It was seen as essential that each step of an investigation be documented with a protocol and annotated with the resulting data, both the original results and any subsequent processed data. Providing information on the cells, reagents, and compounds was also highlighted as an important activity. It should be possible to search and download any protocols or investigation data; however, supporting integrated data analysis within the data warehouse was considered an important longer-term activity, which is currently being elaborated in a second phase requirements analysis.

Outline for the **ToxBank** Data Warehouse based on the requirements: The warehouse is being developed to manage and provide access to all protocols and experimental data across the **SEURAT-1** Research Initiative to support an integrated data analysis. Once a new protocol has been developed, documented and reviewed within the partner's organisation, it can be uploaded to the warehouse. Investigation data can be prepared and reviewed by the **SEURAT-1** investigators using a tool to enter the experimental design, the individual steps of the investigation, each linked to both **SEURAT-1** protocols and the raw or processed data (*Figure 4.52*). The protocols and data loaded can be accessed via a simple free text search or through a browse function that categorises the protocols and data based on common keywords or projects that have loaded the data. This returns summaries of any information matching the query. The protocols or investigation data can then be viewed or downloaded directly along with links to related information, such as the **ToxBank** Gold Compound or Biomaterials wikis (containing information on the standard reference compounds or the biomaterials to be used across the **SEURAT-1** Research Initiative).

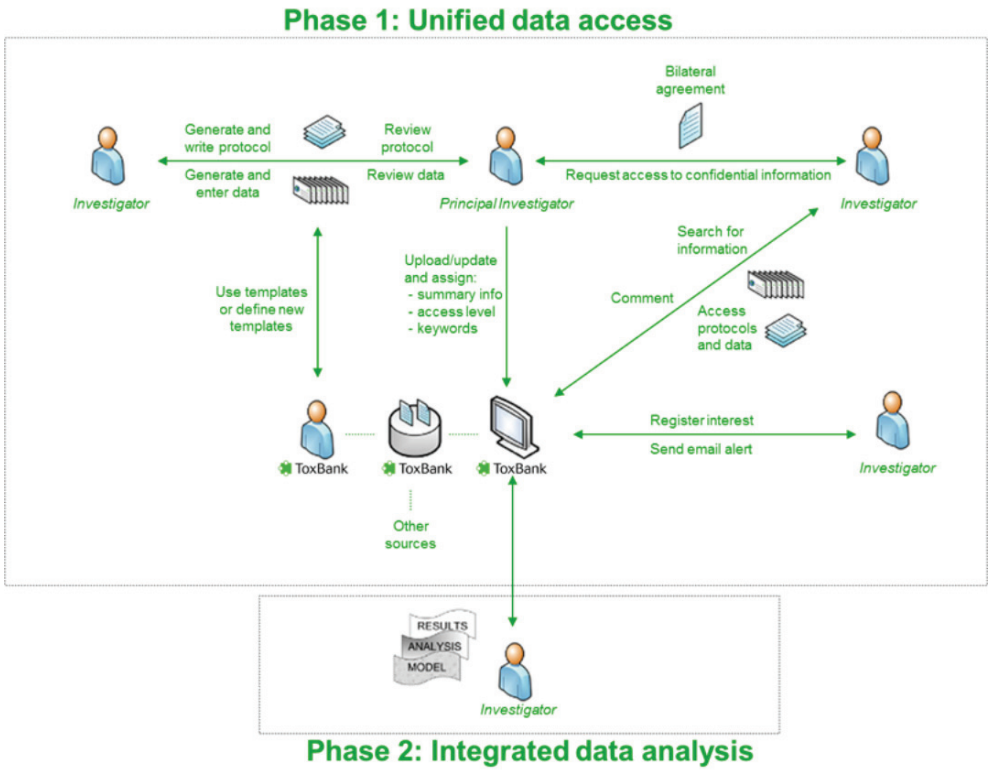

Figure 4.52 *The* ***ToxBank*** *Data Warehouse operations showing the phase I system that is currently implemented alongside a future phase 2 that will include an integrated data analysis.*

Data Analysis Working Group: At the start of the **SEURAT-1** Research Initiative we established a cross-project Data Analysis Working Group (DAWG) open to participation by representatives from all consortia (see also chapter 4.10.4). The DAWG discussed best practices, standards and common approaches for cluster-level data management and analysis in **SEURAT-1** including topics such as vocabularies, protocols, ontologies, statistical analysis, and integrated data analysis. The group also developed ideas and new approaches to data analysis required by emerging research activities carried out in **SEURAT-1**, e.g., the extraction of biomarkers from '-omics' data. Regular virtual discussions are held between the DAWG group members**.**

Design of system architecture: Following the guidance of the detailed requirements output, a system design and architecture that defined the individual information technology components was specified. The **ToxBank** architecture consists of a set of web services, providing access

to protocols and data, a search service, and a Web GUI application, offering user-friendly access to the above functionality. **ToxBank** currently adopts the OpenTox framework design, based on the following technological choices (i) the REpresentational State Transfer (REST) software architecture style allowing platform and programming language independence and facilitating the implementation of new data and processing components; (ii) a formally defined common information model, based on the W3C RDF and communication through well-defined interfaces ensuring interoperability of the web components; (iii) authentication and authorisation, allowing defining access policies of REST resources, based on OpenAM.

Incorporation of systemic toxicity ontology: The reuse and development of relevant core toxicology and neighbouring biological, chemical and medical ontologies is essential for the effective handling of data and information originating from multiple sources, providing interoperability between different systems, providing enhanced search and data mining solutions, and supporting the integration of toxicology, '-omics', clinical and molecular data. **ToxBank** in collaboration with other **SEURAT-1** consortia has created a keyword hierarchy that is incorporated in the warehouse. In addition to its use in facilitating collaborations, the keyword hierarchy is used to support searching, browsing and linking of resources within the warehouse. When information is uploaded, terms can be selected from this hierarchy and linked to protocols and investigation datasets in the warehouse. The keyword hierarchy is currently organised into six main branches: biomaterials, investigative techniques, data and readouts, adverse events, modes-of-action, and standard reference compounds (Gold Compounds').

Implementation of the Data Warehouse through a suite of distributed data marts: To handle all of the data generated in the diverse investigations being performed across the **SEURAT-1** Research Initiative, as well as relevant public data, **ToxBank** adopted the ISA-TAB universal data format to represent the experiments, including the toxicity studies, any chemical analysis, and '-omics' experiements. Data access and upload procedures are defined by the Investigation API. Data is uploaded in ISA-TAB format; data queries are performed with the SPARQL query language. REST operations are available for accessing individual investigations, studies, assays and data files.

Implementation of Data Warehouse operations using web services: The warehouse has a series of operations for managing protocols, data, and searching. These operations have been implemented as a set of distributed web services that make use of existing OpenTox APIs. The specific services implemented are based on the needs of the cluster, as defined in the requirements described earlier. **ToxBank**'s REST resources are instances of the relevant RDF classes.

ToxBank puts special emphasis on data confidentiality. The authentication and authorisation infrastructure, in particular, builds upon what has already been developed and well tested in the OpenTox project and strives to further enhance it.

Searching within the **ToxBank** system is provided as a separate web service that is deployable within an existing web container or as a stand-alone application. It was developed using Java and various open source technologies including Restlet (www.restlet.org/) and elasticsearch (www.elasticsearch.org/). The search service will primarily be accessed by the protocol, investigation, UI services and desktop applications as ISAcreator (https://github.com/ISA-tools/ISAcreator). When protocol or data resources are uploaded, the corresponding service will notify the search service that a new resource is available. The search service will then retrieve the resource and make it available for indexing.

Development of graphical user interface: Web-based graphical user interfaces have been designed, customised and implemented for loading the data into the warehouse as well as accessing the information and model results generated.

Data Entry: A series of forms-based user interfaces have been developed and/or customised for loading experimental data and related descriptions of experimental protocols. To collect investigation data in a consistent manner across the **SEURAT-1** Research Initiative, the **ToxBank** consortium selected to use ISAcreator, an open access tool (http://isatab.sourceforge.net/isahelp/ch03.html). ISAcreator provides a graphical user interface to create a consistently recorded series of data files that include the experimental design and information concerning the overall investigation, information on the experimental steps linked to both protocols as well as raw or processed data files. This tool is essential for entering information in a consistent manner. The tool design as an OSGI compliant framework, allows to extend its functionality by developing custom ontology lookup plugins. **SEURAT-1** specific resources (users and organisations as well as **SEURAT-1** protocols and keywords) are integrated via a custom **ToxBank** plugin (https://github.com/ToxBank/toxbank-isa-plugin), utilising **ToxBank** REST API client (https://github.com/ToxBank/toxbank-api-client), and a **SEURAT-1** specific ISAcreator installer.

The ISA-TAB model allows experimental factors and other properties to be defined as ontology entries that require specific ontologies, facilitating the reuse of ongoing ontology developments in bioinformatics. ChEBI (www.ebi.ac.uk/chebi) and Unit Ontology (http://www.ebi.ac.uk/ontology-lookup/browse.do?ontName=UO) are considered relevant for representing chemical compounds and measurement units; specific ontologies such as Cell Line Ontology (http://bioportal.bioontology.org/ontologies/1245) will be identified and reused when designing templates for raw and processed biological data upload.

Data access and analysis: The protocols, investigation and model results housed in the warehouse are available for access from the web-based user interface. This GUI is a front-end user interface for the repository services defined by the **ToxBank** API. It is a standalone web application allowing users to log in, review existing protocols and investigations, and to upload new protocols and investigations (*Figure 4.53*). The interaction between the users' web browsers and the **ToxBank** UI server relies on standard HTML/CSS/Javascript content, generated dynamically within a Java-based web application framework (the play framework). The **ToxBank** client API is a Java interface to the RESTful **ToxBank** API which is used to communicate with the various repository services. The underlying data driving this content is provided by the repository services via this client. Each user session maintained in the **ToxBank**-UI server contains the user's authentication credentials, task statuses for any of the repository services, and some temporary files for transferring resources between the user's browser and the repository services.

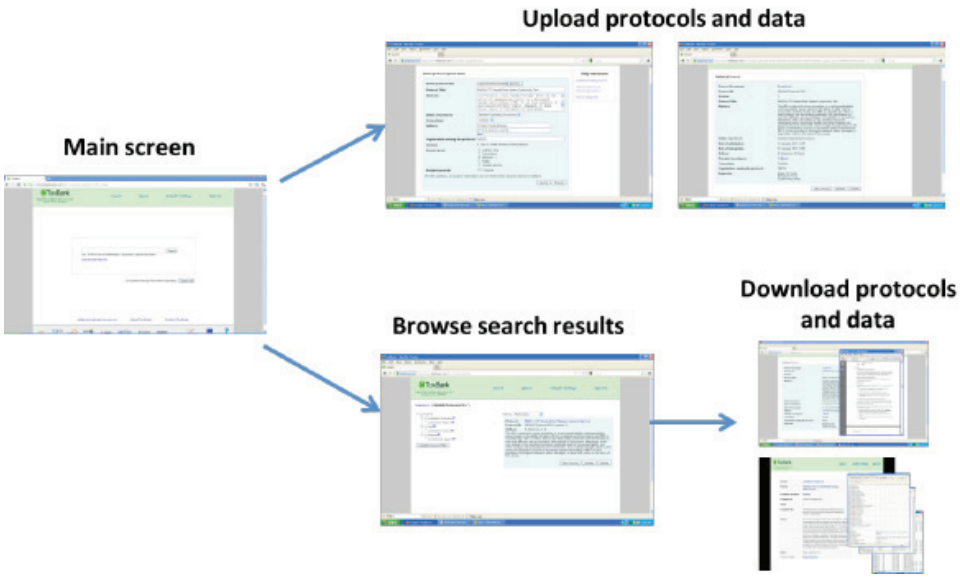

***Figure 4.53 E**xample screenshots from the **ToxBank** Data Warehouse GUI illustrating different scenarios for uploading information and searching the content.*

Integration of Tools for Data Analysis, Mining and Model Building: **ToxBank** will provide an integration of further advanced tools for analysis, model building, visualisation, property calculation, hybrid *in vitro* and *in silico* modelling, regression, classification, and data mining. As discussed earlier, the use and extension of OpenTox APIs within the warehouse will ensure the automatic inclusion of the full extensive suite of algorithms and modelling tools made available under OpenTox, and additional modelling capabilities from any other **SEURAT-1**

partner or external academic or commercial software provider who creates and provides OpenTox API-compliant web services. At this time, the **ToxBank** consortium is investigating consortium and cluster activities and case studies to help understand in more detail use cases for an integrated data analysis based on **SEURAT-1** and public data. Through the DAWG cross-cluster working group, we will also further develop use cases and tool support for an integrated analysis.

Data Warehouse Support Facilities: This task will provide a number of ongoing support tasks to all consortia throughout the runtime of the **SEURAT-1** Research Initiative including refined building of models, biomaterials and compound registration, consultation and customer support. Documentation to support the use of the warehouse as well as the development of protocols and investigation data sets has been developed.

Data resources, tools (e.g., TTC, QSAR), APIs and workflows developed within **ToxBank**, COSMOS and other cluster partners will be incorporated interoperably into **ToxBank** services as they become available.

4.7.4 Innovation

A goal of the **SEURAT-1** Research Initiative is to investigate the applicability of model systems for uncovering chemical-MoA associations and the robustness of the associations across several model systems from 2D cultures of cell line models to primary cell cultures to highly developed bioreactors. In order to achieve this both the descriptions of experimental metadata and the most relevant results need to be standardised with the use of ontologies and the **SEURAT-1** keyword hierarchy as developed by **ToxBank**. Semantic web technologies will enable flexible data mining of the entire dataset repeatedly as new data is generated and submitted to the **ToxBank** Data Warehouse and facilitates creating connections to external data such as the Comparative Toxicogenomics Database (CTD) and the processed data from the DrugMatrix and TG-GATEs repositories. The analysis methods and results of an initial integrated analysis of public data related to the 'Gold Compounds' are discussed in the project-related publication (*Kohonen et al., 2013*).

4.7.5 Cross-Cluster Cooperation

Right at the start of **SEURAT-1**, the first two **SEURAT-1** cross-cluster working groups were established by **ToxBank**: the Data Analysis Working Group (DAWG) and the Gold Compound Working (GCWG). DAWG meetings and communications discussed the expected data analysis requirements of the cluster. The GCWG meetings were held to discuss the approaches to the selection of reference compounds for testing within the cluster, to assemble and analyse the

background knowledge on potential candidates, and to accept or reject candidates as 'Gold Compounds', to be used in cross-cluster testing.

The cross-cluster working group approach proved particularly successful and was adopted and expanded by COACH into other areas during 2012 as a key organisational structure for cluster activities (see also chapter 4.10.2). The working group activities provided very valuable background information and interactions that aided the development of the warehouse design.

Numerous virtual meetings and seminars were organised focused by the priorities and issues of the initial two cross-cluster working groups. These interactions and discussions proved valuable in initiating contacts and developing understanding between different partners from different consortia. Insights and consensus from these interactions provided valuable inputs to **ToxBank** requirements analysis and infrastructure development.

4.7.6 Expected Progress within the Third Year

The currently proposed '21st Century toxicity testing paradigm' is based on a mode-of-action framework that relies on the understanding of biological pathways and mechanisms of action that underlie the toxicity of chemicals *in vivo*. The **SEURAT-1** Research Initiative is developing a MoA-based strategy for animal-free replacements of repeated-dose toxicity testing. Ongoing work from the **ToxBank** infrastructure project is supporting the research activities of the **SEURAT-1** Research Initiative including the selection of standard reference compounds that stratify different MoAs associated with repeated dose toxicity, that are potentially relevant across multiple endpoints and organs, such as liver, kidney, heart and the brain. The standard reference compounds will be used within all consortia to ensure the experimental results can be combined from the different research activities. The **ToxBank** Data Warehouse will house **SEURAT-1** generated results and protocols as well as relevant data from outside the cluster. The warehouse has been developed to enable any future integrated data analysis through the use of RDF and REST-based web services. The warehouse has been designed to support research scientists in the development of a replacement to the current repeated dose toxicity tests; however, as the project develops, more emphasis will be placed on the use of these approaches to support stakeholders from industry and regulatory agencies for risk assessment purposes.

4.7.7 Future Perspectives

The **ToxBank** project establishes critical infrastructure and services functions to all **SEURAT-1** projects providing a centralised and standardised set of data resources, compounds, and biological samples accompanied by standardised operating procedures and guidance. The

provision of quality sources of compounds, cells and tissues for research will promote novel human cell-based assays that will facilitate more accurate evaluation of toxicity. These resources will ensure that the alternative *in vitro* assays developed by research activities in **SEURAT-1** are guided and supported from an early stage of design, to maximise their potential of reaching the pre-validation stage as defined by ECVAM, and eventual validation and regulatory acceptance such as required under REACH. Thus, regulatory agencies are target beneficiaries for this infrastructure. REACH places a significant demand on all businesses operating in the European marketplace involved in the import and manufacture of products involving chemical entities; furthermore companies are required to address the '3Rs' principles and evaluate and potentially use and report on alternatives, wherever possible. Thus, industry is another major target stakeholder for our infrastructure as industry-standard resource facilities such as **ToxBank** are required for safety assessment activity. SMEs particularly will be challenged by regulations as they frequently do not have in-house tools and knowledge resources for the assessment work. We also should have beneficial impact on Cosmetics Europe and other organisations affected by the Cosmetics Directive which places a very strong legislative 3Rs requirement on consumer product companies as all systemic toxicity animal experiments should be replaced starting in 2013.

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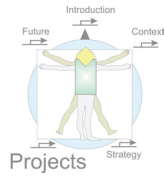

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4.8 COACH: Coordination of Projects on New Approaches to Replace Current Repeated Dose Systemic Toxicity Testing of Cosmetics and Chemicals

COACH

Emmanuelle Da Silva, Bruno Cucinelli

4.8.1 Introduction

COACH is a coordination and support action of the FP7 HEALTH programme which started on 1 January 2011, together with the six research projects of the **SEURAT-1** Research Initiative (presented in the previous sections).

The main role of **COACH** is to:

- facilitate cluster-wide internal cooperation;
- provide strategic guidance with the help of the Scientific Expert Panel;
- prepare and distribute the **SEURAT-1** Annual Reports;
- organise the **SEURAT-1** Annual Meetings;
- coordinate cluster-level dissemination and outreach activities.

COACH provides a central scientific secretariat to the **SEURAT-1** Research Initiative (the '**COACH** Office'), organising the cluster-level interactions and activities and being the main entry point at the cluster level for all organisations, including the funding organisations, i.e., the European Commission and Cosmetics Europe and any external organisation looking to liaise with the initiative (*Figure 4.54*).

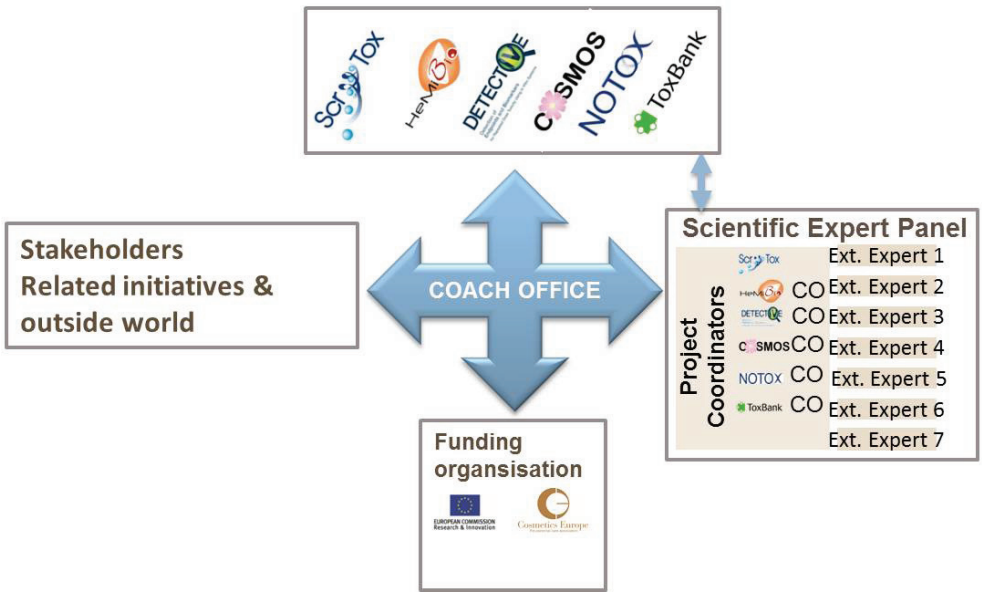

Figure 4.54 *The* ***COACH*** *Office as the central contact for cluster-level activities.*

Each of the seven projects of the **SEURAT-1** cluster is governed by a contractual framework composed of a contract with the European Commission (the FP7 Grant Agreement) and a contract with the cosmetics industry association, Cosmetics Europe. These contracts define 18-month work periods (reporting periods). The first work period finished at the end of June 2012. Each of the seven projects prepared a written report about the work done and results of the first 18 months, and a related common hearing session with independent reviewers was organised by the European Commission and Cosmetics Europe early 2013. The second 18-month work period concludes at the end of December 2013.

The following sections highlight some important achievements of the first and second periods (illustrated in *Figure 4.55* below).

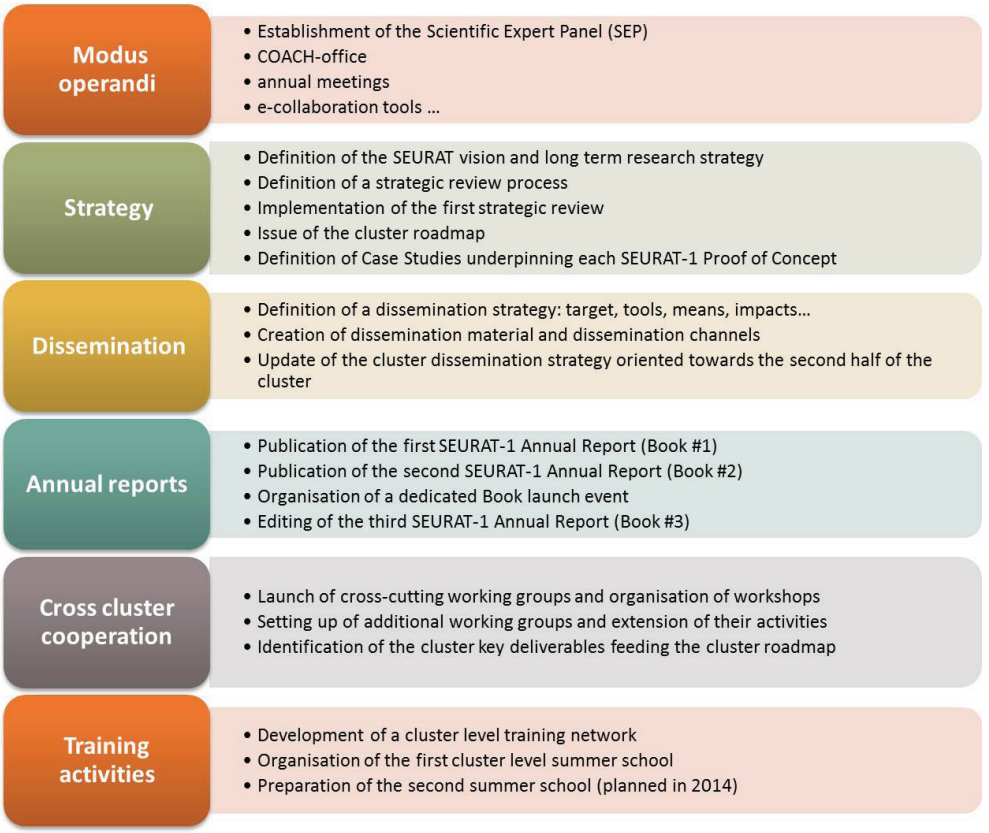

Figure 4.55 *Main cluster-level achievements of the* ***SEURAT-1*** *Research Initiative since the launch of the initiative.*

4.8.2 Cluster-Level Coordination

As with any collaborative research initiative, the starting period for **SEURAT-1** was key to short- and long-term success. At the start of any research collaboration, the partners need to establish the methods, means and common references that will allow them to organise the collaboration in the most efficient and fruitful manner. This was even more important for **SEURAT-1**, in the context of the simultaneous start of six individual research and development projects, which form a cluster of complementary research activities, working towards a common aim. **COACH** played a key role in this specific context. At midterm, the achievements of **SEURAT-1** can be considered successful.

The scientific management and coordination of the **SEURAT-1** Research Initiative is strongly supported by the Scientific Expert Panel (SEP), which plays a key role in providing scientific advice regarding the research work and future orientation of **SEURAT-1**. The SEP is currently

composed of the coordinators of the six cluster research projects plus six external experts. Details about the current SEP members are summarised in *Table 1.1* in the Introduction of this Annual Report (see chapter 1).

Research Strategy, Strategic Review and Roadmap

The SEURAT vision and long-term research strategy were described in the first volume of the **SEURAT-1** Annual Report, issued in September 2011 (*Whelan & Schwarz, 2011*). The research strategy, adopted by the SEP in July 2011, was based on a discussion paper prepared by **COACH** partners University of Tübingen and Joint Research Centre. The strategy describes how the **SEURAT-1** Research Initiative wants to achieve the long-term target of replacing animal testing in human safety assessment, which is the global research target of **SEURAT-1** and beyond.

To enable the SEP to monitor the cluster-level progress made by **SEURAT-1** towards its global objectives, **COACH** proposed a method and plan for performing regular strategic reviews of **SEURAT-1**. Besides this precise objective, the motivations for implementing this plan were also to

- facilitate the engagement and advisory role of the SEP;
- identify critical areas of project interaction;
- establish a high-level roadmap indicating key milestones to serve as a basis for tracking progress;
- provide analysis to aid strategic decision-making.

The strategic review process was prepared by **COACH** partner Joint Research Centre and consists of two main components: (i) a SWOT analysis as a practical tool to better understand how to benefit from strengths and opportunities and how to confront weaknesses and threats at the cluster level; and (ii) the development of a roadmap for monitoring progress at the cluster level. The SWOT analysis was carried out as a brainstorm exercise by **COACH**, the coordinators and the SEP members as well as the Cosmetics Europe Advisory Board members. The feedback was collected and summarised in a report, which was then further discussed by the SEP to identify actions that would improve cluster interactions and achieve a high-level outcome. This exercise will be repeated on an annual basis and, thus, will provide the SEP with a tool to understand whether the efforts made as a follow-up to the first report were fruitful. The cluster-level roadmap as the second part of the strategic review was prepared based on the following steps:

1. Identification of core topics of cross-cluster importance that are critical in achieving the **SEURAT-1** objectives;

2. Identification of the projects and project deliverables that are relevant for each topic;

3. Aggregation of the identified deliverables to determine high-level milestones that define the roadmap for each topic;

4. Assignment of the topics to dedicated working groups and a recommendation that workshops be organised to formulate cluster-level research questions.

The first strategic review carried out by **COACH** with the contribution of the project coordinators was presented during the SEP meeting held in June 2012. The presentation included a detailed description of the cluster-level objectives, the pooled results of the SWOT analysis, an analysis of cross-cluster interactions, and a preliminary outline of the **SEURAT-1** roadmap. Regarding the SWOT analysis, the majority of replies referred to 'strengths and weaknesses' while fewer replies referred to 'opportunities and threats'. Thus, in this first SWOT analysis, participants were apparently more concerned with issues of 'internal origin' rather than of 'external origin'. This 'inward-looking' perspective is understandable considering that the questionnaire was circulated in the first years of **SEURAT-1**. The SEP identified and discussed areas within the cluster that needed more attention, and tried to find ways to benefit from strengths and tackle problems arising from the weaknesses. The SEP proposed, as far as possible, solutions to these areas of concern and some additional activities were initiated. An update of the strategic review and the status of a more detailed roadmap based on the most recent contributions from the coordinators were again presented in a subsequent SEP meeting in November 2012. Further details, including the first **SEURAT-1** roadmap, are given in section 4.10.1 of this Annual Report.

The strategic review is hence performed on a regular basis and is reported formally at the occasion of each SEP meeting. This allows the SEP members to identify potential gaps and weaknesses. Dedicated discussions are also organised on the progress and possible improvements of cross-cluster interactions on the basis of the updated roadmap.

Collaborations with Related Initiatives

The collaboration with related research initiatives and institutions in and outside Europe has been considered important by **COACH** since the start of the **SEURAT-1** initiative. Links were established in particular with: AXLR8 (Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development), EPAA (The European Partnership for Alternative Approaches to Animal Testing), and Tox21/ToxCast (research programmes of the US Environmental Protection Agency). More details about related international research programmes are summarised in chapter 5.2.

The organisation of the workshop '**SEURAT-1** meets Tox21' illustrates the role of **COACH** in supporting collaborations between **SEURAT-1** and related initiatives. The workshop organised by **COACH** partner Joint Research Centre, took place on 25–27 June 2013 in Ispra, Italy, brought together leading scientists from **SEURAT-1** and Tox21 to exchange information on these two major large-scale research initiatives contributing to advancing safety assessment science using non-animal methods. In particular the workshop aimed to map complementary research topics and activities within the two consortia and to identify concrete opportunities for cooperation that can be followed up on a multi-lateral basis by the relevant partners.

The launch of the Annual Report also provides the opportunity for deepening the relationships with other international activities. The second Annual Report was presented at the Euroscience Open Forum (ESOF), which took place in Dublin on 11–15 July 2012. A specific book launch event, to which the press was invited, was organised by **COACH** on 14 July 2012 at ESOF, and research highlights from the first year were presented by representatives from research projects as well as from **COACH**. The launch of this third Annual Report is foreseen in the context of a stakeholder event, which **COACH** is currently organising on behalf of **SEURAT-1** supported by the two main sponsors DG RTD and Cosmetics Europe, in collaboration with EPAA. The event is planned for 5 September 2013 in Brussels and major highlights, as reported in chapter 4, will be presented on this occasion.

Finally, **COACH** is also currently planning to organise its next summer school (2014) jointly with the next ESTIV conference (see the following section and chapter 4.11.1). This is another good example of a fruitful collaboration between complementary initiatives, facilitating the exchanges between the participants in each of the projects, to the benefit of all.

4.8.3 Facilitating Exchanges between the Participants

SEURAT-1 involves over 70 organisations spread across Europe (and some outside of Europe), therefore efficient tools to support remote collaboration are key. **COACH** set up e-collaboration tools at the outset of the initiative, and these have been used intensively since their creation. Besides dedicated mailing lists, **COACH** provides a collaborative web platform, operated by partner ARTTIC, which facilitates the sharing of information and remote collaboration. The private workspace for the **SEURAT-1** partners is accessible for registered users who are involved in the cluster projects, the European Commission and some experts of Cosmetics Europe who signed a special Non-Disclosure Agreement.

The **SEURAT-1** Annual Meetings are the main event for face-to-face meetings of cluster participants. The first two Annual Meetings (March 2011 and February 2012) were organised with the same concept: (i) a plenary session involving a series of keynote speeches about important issues in alternative human safety testing international research, including progress made by the cluster projects; (ii) parallel working groups focusing on specific cross-cluster

topics; and (iii) a panel discussion drawing conclusions from the discussions and providing a common view on future work orientations and priorities of the research initiative. The third Annual Meeting (March 2013) was organised differently, in order to adapt to the evolving cross-cluster cooperation needs of the initiative: it included a new activity called the ***SEURAT-1*** *Market Place*. Held over an afternoon and a morning, this session hosted several parallel discussions, proposed either by the projects, the working groups or by **COACH** to cover certain common cluster issues in more detail (see chapter 4.9.2). There were also short presentations made on scientific progress in connection to a poster session, during which three young scientists were awarded with 'Excellent Poster Awards': Adil El Taghdouini (*HeMiBio*), Simona Kovarich (COSMOS) and Alicia Paini (COSMOS) (extended abstracts are given in chapter 4.9.3).

Another important element of fostering collaborations between the scientists in the different research projects is the organisation of cross-cluster working groups. A detailed overview of these working groups is given in chapter 4.10.2 and activity reports are presented in chapters 4.10.3–4.10.8. To initiate and stimulate the working groups, **COACH** partners Joint Research Centre and University of Tübingen offered workshops during the Annual Meetings as breakout sessions, and also focus work meetings at facilities of the Joint Research Centre in Ispra, Italy, and in Tübingen, Germany (further details are given in the activity reports of the working groups). Furthermore, **COACH** partner Joint Research Centre made a proposal for the definition of Terms of Reference for these working groups; this was first discussed with the coordinators of the six research projects and then submitted for agreement to the Scientific Expert Panel. Based on these agreements, each working group has a clearly defined scope and is coordinated by two co-leaders. Besides actively preparing workshops (see above), **COACH** supports these working groups in organisational matters and has set up dedicated workspace for each working group on the collaborative private web platform to ease communication and collaboration among the working group members.

After defining a homogenised training programme and setting up a training task force composed of representatives from each of the projects, **COACH** held the first **SEURAT-1** Summer School in June 2012. This first training event was key to supporting the integration of the young researchers and it was found to be a suitable environment for scientific exchanges and the creation of long-lasting links between groups working in related research domains. The second summer school will take place in June 2014, most likely in conjunction with the ESTIV conference. It is planned that the second **SEURAT-1** summer school will cover mostly practical sessions while the theoretical topics will be covered under the ESTIV programme. Section 4.11.1 provides further details on the training activities.

4.8.4 Information Dissemination

In order to ensure a good visibility of this research initiative, suitable dissemination material was created and the suitable dissemination channels have been set up:

➠ A consistent visual identity for **SEURAT-1** has been developed (logo, colours, layout of printed and electronic dissemination material, website look and feel, etc.) in collaboration with a professional design company.

➠ A number of information dissemination support materials have been created and distributed since the start of the initiative, including a leaflet, a poster and a 'who's who' booklet, all of which are distributed at each Annual Meeting (and also available online), and a standard PowerPoint presentation.

➠ A public website was set up and went online at the URL www.SEURAT-1.eu. It presents the research initiative, its background and aims, the cluster projects and the involved partner organisations, and promotes the research activities in the field of human safety assessment, in particular regarding alternatives to *in vivo* repeated dose systemic toxicity testing. Dedicated pages present related events, links, publications, job announcements, etc.

➠ The preparation of the first and second **SEURAT-1** Annual Reports was coordinated by **COACH** partner University of Tübingen, who proposed the content structure and contributions required. For each report, the proposed structure and approach was reviewed and endorsed by the Scientific Expert Panel, who contributed actively to the writing and the validation of the book's contents. Partner University of Tübingen collected, reviewed and edited the contributions while ARTTIC conceived the book layout concept in collaboration with an appointed professional designer. The first Annual Report (*Schwarz & Gocht, 2011*) was successfully completed by end of September 2011. Online and printed versions of the second Annual Report (*Gocht & Schwarz, 2012*) were available in July 2012; it was presented at the Euroscience Open Forum (ESOF; see above). About 1,400 copies of each Annual Report were printed and distributed by mail and at relevant conferences (further details on the event are given in chapter 4.11.3).

➠ A dedicated dissemination channel for the Annual Report was created in the form of a mail list, containing over 500 postal addresses of scientists, experts and stakeholders in **SEURAT-1** research results.

➠ The **COACH** partners were very active in promoting the objectives, approach and progress of **SEURAT-1** by participating in close to 30 international conferences and workshops. This strongly contributed to increasing the visibility of **SEURAT-1** on the international scene, hence triggering further interest; this is largely illustrated by an increase of requests to receive the Annual Reports. The events where **SEURAT-1** were present is listed in chapter 4.11.3.

4.8.5 Next Steps

The current work topics on the **COACH** priority list include:

The update of the strategic review of the cluster: At the time of writing this article **COACH** is performing a new SWOT analysis with the aim of presenting the outcome of its strategic review at the SEP meeting on 24 June 2013.

The dissemination strategy: As previously mentioned, one of the important goals of the **COACH** project is to disseminate information about the **SEURAT-1** initiative and to enhance the cluster's visibility. At the outset of **SEURAT-1**, **COACH** had prepared a dissemination plan as a project internal working document, which defined the dissemination objectives and the appropriate means required to reach the dissemination targets. A number of dissemination actions have been carried out since then, leading to excellent visibility of **SEURAT-1** and its recognition as the major European research initiative in the field of alternative human safety testing methods, in particular in the international scientific community. Now, 30 months after the official launch of the initiative, a need has been identified to refocus and prioritise the dissemination strategy more towards the stakeholders of **SEURAT-1**, i.e., the industry, regulators, the public, and policy- and opinion-makers. The research community should of course also remain an important target for information dissemination, exchanges and collaboration. For this purpose, a dissemination strategy paper is currently being prepared by **COACH** and will be presented during the next SEP meeting (24 June 2013). This document aims to define dissemination objectives, means and channels to target these different groups specifically, and to establish accordingly a plan of appropriate concrete dissemination actions. It is planned that this document will be a living tool to be reviewed at each physical SEP meeting, in order to monitor the progress made in its implementation and allow for regular updates to take into account the evolving international context and prioritisation of dissemination actions by the SEP. The status of the dissemination strategy paper and the underpinning actions will be reported in the fourth Annual Report.

The liaison with related initiatives: As detailed above in the section related to the dissemination activities, **COACH** will also focus its efforts on the organisation of events with several renowned initiatives. This includes the joint workshop '**SEURAT-1** meets Tox21' to exchange information, map complementary research topics and activities and identify concrete opportunities for cooperation that can be followed up on a multi-lateral basis by the relevant partners. It is also foreseen that **SEURAT-1** will establish contacts with the IMI Drug-Induced Liver Injury (DILI) programme. The outcome of these actions and events will be reported in the next Annual Report.

The visibility towards the stakeholders of **SEURAT-1**: This will be furthered by the organisation of a stakeholder event in September 2013 in collaboration with EPAA.

The organisation of the second **SEURAT-1** Summer school: This will focus on practical

courses, and may take place in combination with the ESTIV 2014 conference. The programme, outcome and feedback from the participants will also be reported in the next Annual Report.

Preparation of the next phase towards the achievement of the SEURAT long-term goals: The partners and stakeholders of this research initiative consider that **SEURAT-1** is only the first step in a long research effort required to develop alternative solutions for human safety assessment with a view to replacing animal testing approaches. The **SEURAT-1** Research Initiative is reaching the midpoint of its five-year duration. The partners and stakeholders are thinking about the next phase of the required long-term research work: what will be the scope of SEURAT-2, what form of public-private partnership could be envisaged, and how could public and private research funding programmes support these research efforts. **COACH** will keep stimulating the preparation of recommendations and/or proposals for the definition of future research work orientations and accompanying activities, such as certification of the developed technologies and tools, and input into public and private research funding programmes.

Priority work topics for the third period will also address the further development of the achievements made in the past periods, i.e., the efficient operation of the Working Groups, the preparation of the next training activities, and the collaboration with related research initiatives and organisations.

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4.9 Project and Cluster Activities

4.9.1 Project Meetings

Mark Cronin, Barry Hardy, Elmar Heinzle, Jürgen Hescheler, Marc Peschanski, Catherine Verfaillie

***SCR&Tox*:** The *SCR&Tox* consortium members hold a face-to-face meeting every six months and gather at web-conferences on a three-monthly basis. Two face-to-face meetings have taken place in the second year; the first was the *SCR&Tox* six-monthly meeting held in Ispra, Italy, at the JRC premises on 6–7 September 2012. The discussion was focused on achievements in the work-packages dealing with biological and technical resources respectively. This meeting was coupled with an 'Assay Development Preparatory Meeting' with COACH members.

The *SCR&Tox* annual meeting was held in Paris, France, on 20–21 February 2013. Representatives of all work-packages were present, including those who are responsible for the assay development (work-package 3) and the development of the industrial prototype (work-package 4) that will be formally active in the second part of the work programme. Members of the external advisory board were also present. The **SEURAT-1** cluster was represented by Tilman Gocht, a member of the COACH consortium.

Web-conference meetings are organised every three months as a fruitful resource for scientific discussion and risk management.

HeMiBio: A progress report meeting took place on 25–26 June 2012 in Gent, Belgium, and collaborations between different partners were planned on this occasion. A joint meeting with delegates from *SCR&Tox*, NOTOX and DETECTIVE was held directly after the progress report meeting (26 June in Gent), with the aim of identifying common goals in the use of bioreactors for cell culturing and toxicity testing as well as in the development of genetically engineered cells. A report from this workshop is presented elsewhere (see chapter 4.10.8).

The second *HeMiBio* Annual Consortium Meeting was held on 17–18 January 2013 in Barcelona, Spain. A winter school focusing on 'concepts of toxicology and risk assessment' was held beforehand (a summary is given in chapter 4.11.1). Alongside discussions about progress and planning of experiments in the coming year, the annual meeting was also used to define interactions with other **SEURAT-1** projects, which were then further elaborated at the **SEURAT-1** Annual Meeting (see the 'Market Place', below). The meeting was attended by members from the advisory board and by Tilman Gocht as a representative of COACH.

DETECTIVE: The second DETECTIVE General Assembly took place from 6–7 February 2012 in Lisbon, Portugal. Progress in the different work-packages was reported to the consortium, and future activities were planned. The advisory board as well as one COACH representative (Tilman Gocht) attended the meeting.

Several meetings of sub-groups were organised over the course of the year in order to plan joint experiments and detailed analysis of the data. Proteomics-specific challenges in data storage and analysis were discussed at a meeting on 6 March 2012 in Dortmund, Germany. DETECTIVE partner Roche organised training on impedance measurements and the Cellavista imaging system on 28–30 March 2012 in Penzberg, Germany. Another meeting took place on 30 May 2012 in Maastricht, The Netherlands, to discuss the '-omics' experimental setup for all three cellular systems (liver, kidney and heart) and to coordinate sample handling and collection. First results were then discussed during follow-up teleconferences on 4 July and 3 September 2012.

COSMOS: A COSMOS meeting was held in connection with the 15th International Workshop on Quantitative Structure-Activity Relationships (QSAR2012) on 18 June 2012 in Tallinn, Estonia, with a majority of the COSMOS consortium partners attending.

The six-monthly General Assembly meeting for 2012 took place at the University of Bradford, United Kingdom, on 13–14 September 2012. It was preceded by a half-day symposium on 12 September 2012 with external guests discussing 'A Roadmap to Navigate from Databases to Adverse Outcome Pathways'.

At the end of the second project year the Third Annual General COSMOS Meeting was held on 4–5 March 2013 before the **SEURAT-1** Annual Meeting in Lisbon, Portugal, to review the second year results, plan the next steps and discuss specific topics within the work-package groups. Furthermore, COSMOS delegates also discussed database interactions with ToxBank. At the **SEURAT-1** Meeting, COSMOS contributed substantially to the poster session, demonstrated the COSMOS database, KNIME workflows and webportal to the other cluster projects, and contributed to other Working Group sessions, including the Biokinetics Working Group co-lead by COSMOS.

In order to determine the needs and requirements of stakeholders, COSMOS held an interactive workshop at a Cosmetics Europe Systemic Toxicity Task Force Meeting on 15 May 2012 in Brussels, Belgium. The purpose of the workshop was to raise the profile of the COSMOS project, disseminate results from COSMOS, highlight upcoming work, evaluate databases and models currently in use, gain opinions from the Systemic Toxicity Task Force regarding COSMOS, and to understand expectations and needs of stakeholders with regard to the future direction of the project. COSMOS received invaluable comments from the industry regarding the use and practice of computational toxicity and different approaches to consider during the further development of methods and tools in the project.

NOTOX: The third NOTOX progress meeting took place on 11–12 June 2013 in Stockholm, Sweden, and was hosted by the Karolinska Institute. Tilman Gocht from COACH and Cosmetics Europe representative Yeyejide Adeleye attended this meeting. Various decisions on future collaborative work and publications were taken.

The fourth NOTOX progress meeting was hosted by The National Center for Scientific Research in Strasbourg, France, on the 4–5 March 2013. This meeting was attended by Cosmetics Europe representative Yeyejide Adeleye. In addition to progress reports, the major focus was on NOTOX publications and planning of long-term repeated dose multi-omics experiments.

4.9.2 Cluster Meeting of the SEURAT-1 Research Initiative

The COACH Team

The third Annual Meeting of the **SEURAT-1** Research Initiative was held on 6–7 March in Lisbon, Portugal. Ian Cotgreave, co-chair of the **SEURAT-1** Scientific Expert Panel (SEP), welcomed the participants and reminded everyone of the expectations expressed at the **SEURAT-1** kick-off meeting two years ago, asking the audience which additional efforts should be encouraged at this crucial mid-term meeting to achieve the cluster objectives. Bernard Mulligan (DG RTD) opened the meeting by emphasising the uniqueness in this public-private partnership, with equal contribution from the Commission (FP7, Health Programme) and Cosmetics Europe. He observed how the goal of the 3Rs in society was developing from a primarily focus of protecting laboratory animals into an additional ambition of improving human safety assessment. **SEURAT-1** fully embraces this. However he encouraged the **SEURAT-1** partners to further collaborate to identify potentially redundant overlaps and transform them into complementarities, and that all individual projects should put a stronger focus on the common cluster objectives. Russell Thomas (SEP) gave an invited plenary lecture on biokinetics in the design and interpretation of *in vitro* experiments to obtain the essential relevance in the application to safety assessment. He mentioned the strong regulatory, societal and economic issues being addressed by **SEURAT-1** and related projects in the United States, to innovate in the safety assessment of chemicals without use of animals.

The three **SEURAT-1** Proof of Concepts were the central theme of this meeting. Brigitte Landesmann (JRC) talked about the first, being the theoretical description of a mode-of-action (MoA). She described the work in progress within the Mode-of-Action Working Group identifying relevant MoAs for the different **SEURAT-1** organs. She described in more detail the experience of MoA development for liver fibrosis and steatosis as summarised in a recent JRC report. Maurice Whelan (COACH, JRC) introduced the second Proof of Concept, inviting all project partners to participate in the development of systems for predicting toxicity. He stressed the need to recognise how mechanistic understanding can be used to combine suitable methods to achieve a prediction goal. Andrew White (Cosmetics Europe) introduced

the third Proof of Concept, describing the two planned case studies as proposed by the Safety Assessment Working Group, one that is data-rich involving a read-across based on integrating structural properties and *in vitro* data, and one data-poor study to understand how to pragmatically build up a chemical safety assessment.

In the afternoon session Elisabet Berggren (COACH, JRC) gave a short presentation on the follow-up to the **SEURAT-1** first strategic review and roadmap, followed by the individual project reviews: Susanne Bremer reported back from *SCR&Tox* on behalf of the coordinator; Catherine Verfaillie illustrated the highlights from the *HeMiBio* progress; Jürgen Hescheler reported on DETECTIVE; Mark Cronin on COSMOS; NOTOX progress was presented by Elmar Heinzle and finally Barry Hardy talked about ToxBank activities.

The **SEURAT-1** Market Place took place in the afternoon and on the morning of the second day. The Market Place hosted several parallel discussions, proposed either by the projects, the working groups or by COACH to cover certain common cluster issues in more detail. There were also short presentations made on scientific progress in connection to the poster session. The plenary re-convened with feedback from the Market Place events and opinions on whether the format had facilitated further progress.

Rob Taalman (Cosmetics Europe), representing one of the **SEURAT-1** sponsors, came to the podium in the final outlook session of the meeting, where he pointed out that the total ban on animal testing under the Cosmetics Directive would enter into force the following Monday, 11 March 2013. He reiterated to the audience that practical solutions to carry out safety assessment without the use of animals are needed now and the partners must take their responsibilities seriously and make every effort to achieve the common **SEURAT-1** goals. The first review, after 18 months, had just been finalised and the next external review was now planned to take place together with the annual meeting in 2014. At that point clear progress towards the common cluster objectives is expected.

Before the closure of the meeting each coordinator had the opportunity to openly reflect on the challenges in the coming year. Susanne Bremer (*SCR&Tox*) informed the audience that they are currently evaluating which 'pathway assay' to choose as defined in the work programme and whether it can serve as a basis for a case study for the second Proof of Concept. She pointed out that traditional validation approaches would not be suitable for pathway-based methods and it will be necessary to further investigate how to address this issue. Catherine Verfaillie (*HeMiBio*) spoke about how that they had chosen fibrosis for further testing and they still needed to evaluate which cell system would be most suitable for continued testing. During the Market Place *HeMiBio* had discussed at length how to include biokinetics in their project and also how to collaborate with other projects. Jürgen Hescheler (DETECTIVE) explained that they now had a large amount of testing data to submit for biostatistical evaluation and based, on this analysis, they would further reflect on how to contribute to a case study. The natural step forward would be to build prediction models based on the results and test them

on a group of structurally similar substances. Mark Cronin informed the group that COSMOS was about to achieve two key deliverables at project level: the COSMOS database and the report on a TTC approach extended to target organ toxicity of cosmetic ingredients. Case studies currently discussed within COSMOS are transparency of TTC, dermal penetration and Molecular Initiating Events for fibrosis and steatosis (LXR receptor binding already made). Elmar Heinzle (NOTOX) explained that they applied a systems biology approach to their experiments and that they had already included *in vitro* to *in vivo* extrapolation in their models. Steatosis will be the case study they will further address within the project. Emilio Benfenati (ToxBank) encouraged partners to provide ToxBank with all their data, because that will be the source of the results collected during **SEURAT-1** once the projects are finalised. There is already a lot of data available in the ToxBank wiki on the gold compounds and the Data Warehouse was just about to be opened up for use of all partners for data storing. Maurice Whelan, who chaired the final session, thanked the coordinators and all the partners for their collaborative spirit and hard work.

Derek Knight, SEP co-chair, introduced the young scientists that were presented with "Excellent Poster Awards":

- Adil El Taghdouini (Vrije Universiteit Brussel, *HeMiBio*): Gene expression and microRNA profiling of human quiescent and activated hepatic stellate cells
- Simona Kovarich (Soluzioni Informatiche sri, Vicenza, COSMOS): Molecular modelling to predict and understand chemical toxicity in the AOP framework – case study: MoA from LXR activation to liver steatosis
- Alicia Paini (Joint Research Centre, Ispra, COSMOS): Human Bioaccumulation Potential simulated in R and implemented in the KNIME Interface

The extended abstracts are given in the following section. The awards were sponsored by Cosmetics Europe, and provided the possibility for the winners to attend a scientific conference of their own choice.

Ian Cotgreave closed the meeting by reminding everyone about the unique opportunity to be a part of the **SEURAT-1** project and to consolidate all efforts, collaborate and together achieve the common goals of **SEURAT-1**.

4.9.3 Young Scientist Poster Award

In total, 32 posters covering diverse research activities in the different projects of the **SEURAT-1** Research Initiative were presented at the Annual Meeting. The e-versions of the posters are published on the **SEURAT-1** public website (www.seurat-1.eu). The poster award committee selected the three best posters, and the awardees present their work in the following extended abstracts (in alphabetical order).

4.9.3.1 Gene Expression and microRNA Profiling of Human Quiescent and Activated Hepatic Stellate Cells

Adil El Taghdouini[1,], Mar Coll*, Inge Mannaerts, Carola Millán, Mustapha Najimi, Etienne Sokal, Juan Caballería, Pau Sancho-Bru*, Leo A. van Grunsven**

** Authors contributed equally to the work*

Introduction

In a healthy liver, hepatic stellate cells (HSCs) represent approximately 8–15% of the resident cells and are strictly located in the space of Disse, a virtual space between the hepatocytes and the endothelial cells of the liver sinusoids (LSECs). Besides their well-known role in the regulation of extracellular matrix and retinoid homeostasis, there is also evidence that HSCs can regulate the sinusoidal blood flow and stimulate angiogenesis. In addition, HSCs secrete a whole range of cytokines, growth factors and lipoproteins and have been identified as being key players in fibrogenesis (*Friedman, 2008*). Fibrosis, or scarring of the liver, is a chronic wound-healing response that recruits a range of cell types and mediators to intercept the injury caused by viral infections, auto-immune, cholestatic and metabolic diseases as well as drugs or alcoholic-induced injury. Cirrhosis of the liver, characterised by replacement of liver tissue by scar tissue and regenerative nodules, ultimately leading to loss of liver function, results from advanced fibrosis (*Bataller & Brenner, 2005*).

A main event during fibrogenesis is the activation of HSCs during which they transdifferentiate from cells with a quiescent phenotype into cells with a fibrogenic-myofibroblast-like phenotype. *HeMiBio* proposes to generate a liver-simulating device, reproducing the heterotypic interactions between parenchymal (hepatocytes) and non-parenchymal cells of the liver that could serve to test the effects of chronic exposure to cosmetic ingredients. In this specific set-up, the identification of potential pro-fibrotic compounds will mainly be based on their ability to induce HSC activation, making correct monitoring of human HSC activation of the utmost importance. In order to fully characterise the transcriptional changes occurring during *in vitro* activation of human HSCs, as well as to identify human liver cell type specific markers, we performed gene expression and miRNA profiling of quiescent HSCs (qHSCs) and fully culture-activated HSCs (aHSCs).

Approach

Following a collagenase digestion of the liver and 50g centrifugation steps to discard hepatocytes, qHSCs and LSECs were sorted from the resulting non-parenchymal cell suspension using flow cytometry. Culture aHSCs (passage 4) were obtained after subsequent passaging of Nycodenz®-isolated HSCs. A strong enrichment in PDGFrB expression in the

resulting HSC populations compared to LSEC (>100 fold) and hepatocytes (>20 fold), indicate a successful isolation of HSCs from human livers using our procedure. In total, mRNA of aHSC, qHSC (both N=9) and LSEC (N=3) from corresponding patients was analysed for gene expression profiling using Genechip HG-U219. Affymetrix gene expression data were normalised using the robust multi-array algorithm using a custom probe set definition that mapped probes to 18567 Entrez Gene lds (HGU219_Hs_ENTREZG). Genes with a coefficient of variation lower than 0.03 were eliminated, resulting in a set of 13925 genes. For the detection of differentially expressed genes, a linear model was fitted to the data and empirical Bayes moderated statistics were calculated using the limma package from Bioconductor. Adjustment of p-values was done by the determination of false discovery rates (FDR) using the Benjamini-Hochberg procedure. Genes representing a fold change of 1.5 or greater and a moderated p-value <0.05 were considered as differentially expressed.

MicroRNA (miRNA) expression profiling of aHSCs (N=4), qHSCs (N=4) (also from corresponding patients) and hepatocytes (N=2) was performed using TaqMan® Array Human MicroRNA A+B Cards. Expression levels of 758 miRNA of quiescent and activated HSC samples were obtained by Taqman qRT-PCR analysis. The abundance of each miRNA in a total RNA sample was normalised to the level of the RNU6 in the same sample (computation of -deltaCt). Fold changes were computed from the means of each group and their statistical significance was established at p≤0.05 (t-student).

Results and Discussion

From a selected heatplot (*Figure 4.56, left*), it is clear that although not all patients have identical expression profiles, clear trends exist between the different cell types. Besides confirming generally used activation markers in human HSCs, such as ACTA2, COL1A1 and LOX, this analysis also identified potentially new activation markers, for example gene A and B (*Figure 4.56, right*), which show an average 32 fold increase in expression upon activation (>5 dCT) (data not shown). In the heatplot below, one can also see one qHSC patient sample (indicated by a star) that has a much higher expression level of both the classical and newly identified activation markers compared to the other qHSC samples, suggesting that this patient had HSCs that were less quiescent.

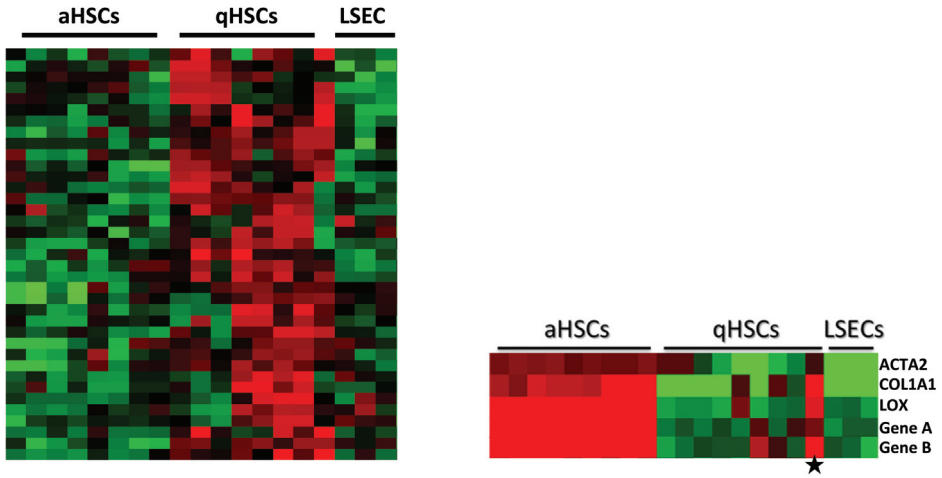

Figure 4.56 *Heatplots of mRNA profiling of human aHSCs, qHSCs and LSECs (left) and of classical and potentially new activation markers in aHSCs, qHSCs and LSECs (right). The heatmaps show significantly up-regulated genes during in vitro HSC activation that are not expressed (or at relatively low levels in LSECs). Red and green colours indicate high and low expression, respectively. The star symbol highlights a qHSC sample that has an expression profile resembling more that of the aHSCs.*

Using Ingenuity Pathway Analysis (IPA), we identified relevant biological pathways related to the differentially expressed genes between qHSCs and aHSCs. As expected, the top enriched canonical pathway overrepresented among differentially expressed genes is 'Hepatic Fibrosis/Hepatic Stellate Cell activation'. Using IPA, we further identified a set of 90 differentially expressed genes that act as upstream transcriptional regulators of a whole range of pro-fibrogenic genes. This set contains genes known to play a pivotal role in fibrosis, such as CTGF, but also genes that have never been described in the context of fibrosis and/or HSCs. We are currently in the process of identifying upstream regulators that are relevant for human disease by characterising their expression pattern in liver tissue samples of healthy and fibrotic/cirrhotic subjects.

In accordance with the changes observed in gene expression, culture activation of HSCs was associated with important transcriptional changes at the miRNA level. Striking is the strong differences in miRNA expression seen between hepatocytes and HSCs (*Figure 4.57*). Integration of both data sets (Gene expression and miRNA profiling data) indicates that 63% of the differentially expressed genes are predicted to be related to the deregulated miRNAs. Measuring the expression levels of newly identified miRNAs that can regulate large networks of relevant genes during human HSC activation might be of interest when monitoring the activation process in the hepatic microfluidic bioreactor.

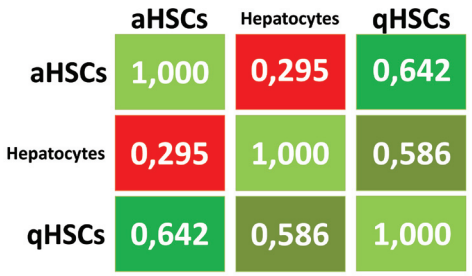

Figure 4.57 *MicroRNA expression profiling in hepatic cells. Distance matrix of miRNA expression in aHSCs (N=4), qHSCs (N=4) and hepatocytes (N=2).*

Conclusions

This expression-profiling experiment validated numerous well-established activation markers in human HSCs, such as COL1A1, ACTA2 and LOX. However, when compared to microarray data of mouse HSCs (*De Minics et al., 2007*), some marked differences exist between human and mouse expression profiles, suggesting that we should be careful when extrapolating mouse HSC data. MicroRNA data analysis showed a massive miRNA upregulation in activated compared to quiescent HSCs, supporting the idea that miRNAs could play an important role during *in vitro* activation of human HSCs (*Chen, 2009; He et al., 2012*). In conclusion, we developed an efficient isolation protocol of human qHSCs which allowed us to compare the gene expression and miRNA profiles of qHSCs and aHSCs. We confirmed several established activation markers but also identified new HSC- and activation-status specific markers to be used for the activities within *HeMiBio*, such as isolating HSCs from differentiated pluripotent stem cell cultures or monitoring the activation status of HSCs in bioreactor cultures.

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4.9.3.2 Molecular Modelling to Predict and Understand Chemical Toxicity in the AOP Framework - Case Study: MoA from LXR Activation to Liver Steatosis

Simona Kovarich, Arianna Bassan, Mark T.D. Cronin, Elena Fioravanzo, Candida Manelfi, Andrew P. Worth, Chihae Yang

Introduction

Within the Mode-of-Action/Adverse Outcome Pathway (MoA/AOP) framework addressing repeated dose toxicity, the **SEURAT-1** Research Initiative supports the development of integrated tools for predictive toxicology (including theoretical, computational and experimental *in vitro* models) aimed at extending the knowledge of toxicological processes associated with repeated dose target organ toxicity. Among the toxicological MoAs defined and documented by the **SEURAT-1** Research Initiative (see report of the Mode-of-Action Working Group, section 4.10.5.2), the 'MoA from Liver X Receptor (LXR) Activation to Liver Steatosis' was selected here as a case study to employ the molecular modelling approach to predict and understand potential liver toxicity of chemicals acting through the LXR pathway. The molecular initiating event identified in this MoA is the binding to the nuclear receptor LXR and its activation by appropriate ligands.

Here we present the results of a preliminary molecular modelling study of the binding of selected ligands to the two isoforms of LXR, i.e. LXRα and LXRβ. The present study had two main objectives:

1. Verify whether molecular modelling approach can be applied to study LXR binding potency;

2. Compare the ligand binding domain (LBD) of the two isoforms of the receptor, in order to verify whether molecular modelling is suitable for modelling LXRα/β subtype-selectivity.

This preliminary study will be a useful element in efforts to verify the postulated MoA for LXR-activated liver steatosis (see section 4.5.3, category formation as part of the COSMOS project report).

Approach

Molecular modelling is a powerful computer-based technology that allows for studying, describing and modelling the behaviour of molecular systems; in particular molecular modelling tools can be used to explore the potential interaction between a small molecule and a complex macromolecule. Molecular modelling has been widely used in pharmaceutical

discovery for more than 50 years. Applying tools developed for drug discovery to predict the toxicity potential of chemicals requires an optimisation of the methods that takes into account the analogies and differences between the two frameworks.

In the present study, molecular modelling was applied to analyse the interactions of the nuclear receptor LXR (both α and β isoforms) with selected synthetic ligands, including T0901317, which is the LXR agonist proposed by the **SEURAT-1** Gold Compound Working Group as the reference chemical for liver steatosis (see report of the Gold Compound Working Group, section 4.10.3.3). In particular the molecular docking technique was employed. Molecular docking is a method to predict the preferred orientation of one molecule to a second when bound to each other to form a stable complex. The theory behind molecular docking lies with the enzyme-substrate recognition process. The problem can be thought as a 'lock and key' concept. In this problem, the orientation of the ligand (small molecule) is 'fitted' to the receptor of interest. The programs 'adjust' the conformation of the ligand and, in some cases, the conformation of the side chains of the receptors binding site (site of interaction) to see how they fit each other. Available 3-dimensional (3D) models of the ligand binding domain (LBD) of LXRα and LXRβ were retrieved by searching the Research Collaboratory for Structural Bioinformatics Protein Data Bank (*RCSB Protein Data Bank, 2013*). The homology analysis of the LBD of different proteins was performed by using the Basic Local Alignment Search Tool (BLAST) (*Schrödinger, 2012a*). Docking studies of T0901317, using the structures of LXRα and LXRβ, were also carried out to further explore ligand-binding conformations and molecular interactions (*Schrödinger, 2012b; c*). Finally, the MM-GB/SA scoring technique (*Schrödinger, 2012d*) was used to calculate binding energy of the docking binding poses. Binding energy is the mechanical energy required to disassemble a whole into separate parts. A bound system typically has a lower potential energy than the sum of its constituent parts; this is what keeps the system together. Often this means that energy is released upon the creation of a bound state.

Results and Discussion

17 3D models of the LBD of LXR (murine LXRα and human LXRα/β co-crystallised with different synthetic ligands) were retrieved from the RCSB PDB and analysed.

To perform a comparison of the two isoforms of the receptor, the sequences of the LBD of LXRα and LXRβ were aligned and analysed with BLAST (*Schrödinger, 2012b*). The LBD of the two receptors showed a high degree of homology (78%) and 100% of sequence identity when only the ligand binding pocket (LBP), i.e. the region where the ligands interact, was considered. The structures of LXRα/β co-crystallised with T0901317 were then superimposed (*Schrödinger, 2012b*) and the resulting tertiary structures in the LBP were identical. The details of the interactions between the receptors and the ligand are represented in *Figure 4.58*. T0901317 is mainly bound by hydrophobic interactions between its trifluoromethyl groups and the protein.

X-ray crystallography determined structures of LXR complexes and revealed two possible binding poses of T0901317 in the LBP of LXRα and LXRβ, i.e. binding pose A (*Figure 4.58a*) and B (*Figure 4.58b*), respectively. This can be explained by the large size of the ligand-binding pocket of the receptor. A docking study was then carried out to study the possible binding poses of T0901317. The results obtained by the docking algorithm showed that the most stable binding pose of T0901317 in both LXRα and LXRβ identified by more negative values of the docking score – *Table 4.5*) is the one experimentally retrieved in LXRβ (i.e., binding pose B).

Prime/MM-GBSA (*Schrödinger, 2012d*) calculations of the binding energy of the docking poses of T0901317 in LXRα and LXRβ confirmed these results (*Table 4.5*).

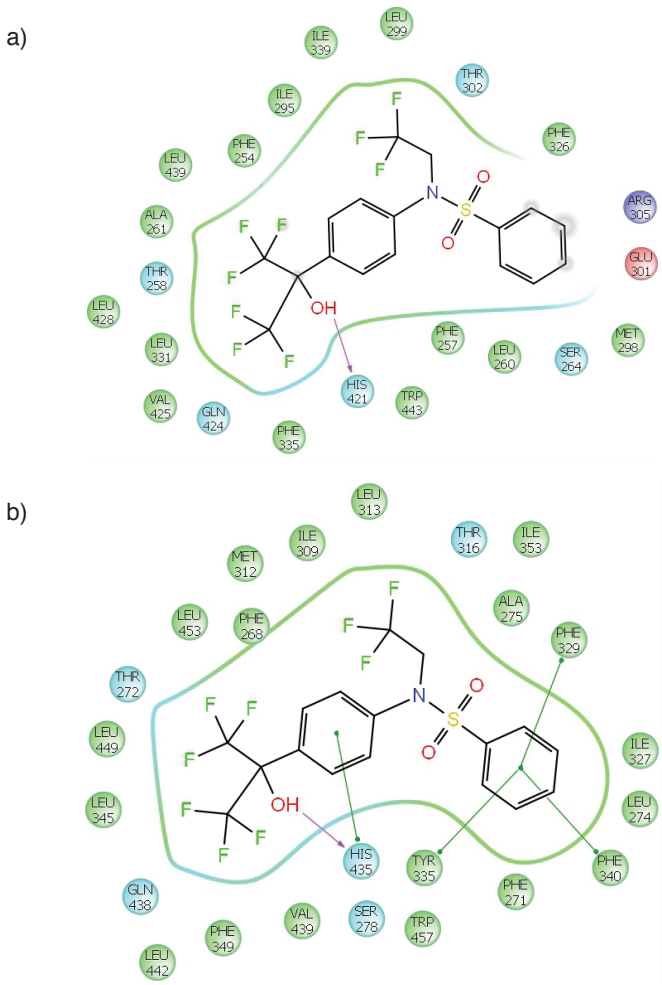

Figure 4.58 *Ligand interaction diagrams of T0901317 in the ligand binding pocket of LXRα (a) and LXRβ (b).*

Table 4.5 *Results of docking studies.*

Crystal complex structure	Docking (Glide score)	DG binding (Kcals/mol)
LXRα-T0901317	-7,5 / -9 (binding pose A / B)	-86 / -90 (binding pose A / B)
LXRβ-T0901317	-10,3 (binding pose B)	-100 (binding pose B)

Conclusions

Molecular modelling methods offer one of several complementary approaches to evaluating the risk of chemicals to human health. Considering that:

- X-ray crystallography 3D structures of several LXR complexes are available;
- Several experimental data for LXR binding affinity are available;
- Experimentally determined X-ray crystallographic binding poses are confirmed by docking studies, which represents a preliminary validation of the computational model (3D structures, docking, and energy calculation algorithm and protocol);
- Preliminary docking studies show high docking score values, indicators of good skill of the computational model to mimic ligand-receptor interactions;

we can conclude that LXR complexes are suitable for studying LXR binding potency. However, since it was demonstrated that LXRα/β have no fundamental differences in the ligand binding pocket, molecular modelling is not suitable for modeling LXRα/β subtype-selectivity.

References

RCSB Protein Data Bank (2013): http://www.rcsb.org/pdb/home/home.do (*accessed 23 April 2013*).

Schrödinger (2012a): BLAST alignment tool provided by Prime, version 3.1. http://blast.ncbi.nlm.nih.gov/Blast.cgi (*accessed 23 April 2013*).

Schrödinger (2012b): Protein Structure Alignment provided by Prime, version 3.1. www.schrodinger.com (*accessed 23 April 2013*).

Schrödinger (2012c): Glide, version 5.8. www.schrodinger.com (*accessed 23 April 2013*).

Schrödinger (2012d): Prime, version 3.1. www.schrodinger.com (*accessed 23 April 2013*).

4.9.3.3 Human Bioaccumulation Potential simulated in R and implemented in the KNIME Interface

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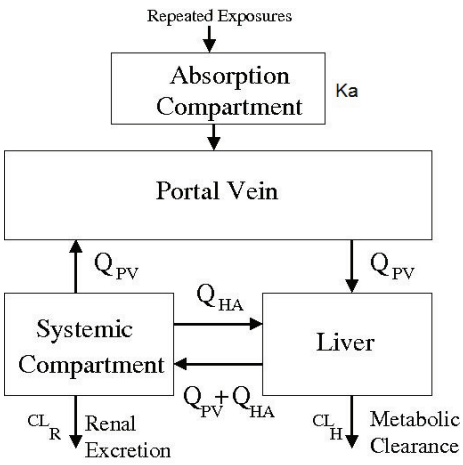
Introduction

Bioaccumulation is the process by which substances not readily broken down or excreted can build up in living tissues (usually fat tissues). In the bioaccumulation process there is an increase in the concentration of a chemical in an organism, compared to the chemical's concentration in the surrounding environmental media to which the organism is exposed (i.e., air, water, soil, food). Bioaccumulation is an important consideration in the assessment of long-term toxicity. Accordingly, it is an element in prioritisation strategies under a range of regulations (e.g., *US EPA, 1998; CEPA, 1999; ECHA, 2008*). Traditionally, bioaccumulation potential has been assessed in aquatic or terrestrial organisms, but not directly in humans. To address this shortcoming, *Tonnelier et al.* (*2012*) developed an approach for predicting human bioaccumulation potential based on the use of a simple PBTK model, coded in the Symcyp software. This generic physiologically based toxicokinetic (PBTK) model (*Figure 4.59*), was able to predict the bioaccumulation potential of a chemical, expressed as the human bioconcentration factor (hBCF), based on selected physicochemical parameters, *in vitro* human liver metabolism and plasma-protein binding data, minimal renal excretion and a constant exposure scenario. In principle, bioaccumulative chemicals can include substances in cosmetics and personal care products, as well as pesticides, plasticisers and degreasers. One difficulty in evaluating the hBCF is the multiple sources of exposure. Furthermore, hBCF cannot be simply defined as the ratio of the concentration in blood and in water. It has been suggested that bioaccumulation should refer to an increase in blood concentration with repeated exposures (*Figure 4.59*).



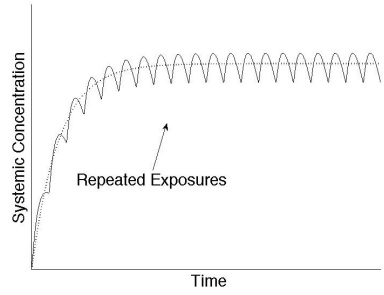


Figure 4.59 *Schematic representation of a three-compartment PBTK model and the increase in blood concentration of the chemical with repeated exposures (adapted from Tonnelier et al., 2012).*

To promote the wider use of the PBTK model, we have re-implemented the model by using open source methodology, and we have illustrated its application to selected chemicals. The results could be used to prioritise chemicals for the further assessment of their potential long-term toxicities.

Approach

Following *Tonnelier et al.* (*2012*), we have re-written the PBTK model in R, and re-implemented the model as an open source KNIME workflow (*Figure 4.60*). This model was designed to incorporate not only the chemical properties of the compounds, but also the processes that tend to decrease the concentration of the compound, such as metabolism. The KNIME workflow consists of several nodes: (i) a database connector (with biophysical and compound-specific properties, such as blood to plasma ratio, unbound fraction of chemical in plasma, etc.) with a query filter node to select the values for the simulated chemical, in the present work the MySQL database was used; (ii) an XLS reader node that provides the model input parameters (e.g., flow rates, organ volume); (iii) an R node where the PBTK model is described mathematically; (iv) an R view node for output.

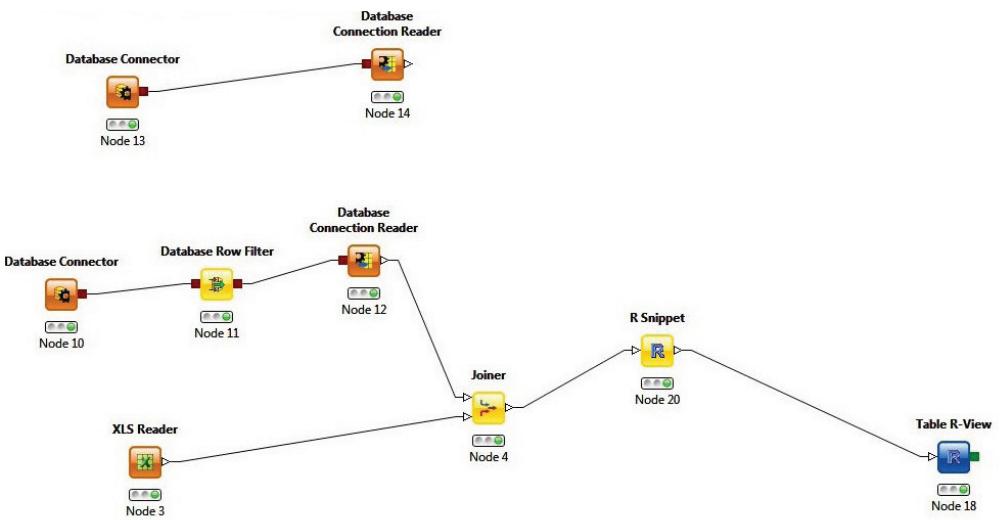


Figure 4.60 *Representation of the KNIME workflow for the PBTK (human bioaccumulation) model.*

Results

The outputs of the KNIME workflow, are: (i) a graphical figure expressed as concentration versus time; (ii) the systemic concentration (C_{sys}) at steady state of the selected compound; and (iii) the human bio-concentration factor (hBCF). The calculated hBCF values were used to rank the 94 chemicals listed in *Tonnelier et al.* (*2012*), which were recalculated using the newly developed KNIME workflow. The hBCF values obtained are within the same order of magnitude as those obtained previously with Symcyp, with the single exception of PFOS. The ranking of the chemicals varies slightly when compared to previous ranking reported by *Tonnelier et al.* (*2012*), as shown in *Table 4.6*.

Table 4.6 *Ranking of chemicals based on hBCF values generated by the KNIME implementation of the PBTK model and the original model of Tonnelier et al. (2012).*

KNIME ranking	*Tonnelier et al.* (*2012*) ranking
Emamectin	PFOS
PCB155	Emamectin
PCB153	Buprofezin
PCB80	PCB80
PCB77	PCB77
PCB136	PCB153
Buprofezin	PCB155
Fenvalerate	PCB136
PFOS	Fenvalerate
Bentazone	Bentazone
Dichlorophenoxy	DDT
Parathion	Parathion
Cyprodinil	Cyprodinil
DDT	Pyraclostrobin
Pyraclostrobin	Dichlorophenoxy
Bromacil	Fipronil
Fipronil	Thioridazine
Warfarin	Warfarin
Thioridazine	Bromacil
Fenoxycarb	Fenoxycarb

Conclusions

The hBCF values obtained in the present study can be used to rank chemicals according to their predicted bioaccumulation potential, and can assist in priority setting for the further assessment of long-term toxicity. Furthermore, this open source approach will help to establish a general procedure for performing *in vitro–in vivo* extrapolation (IVIVE) and being able to move from realistic *in vitro* concentrations to dose–response relationships without the use of animal experiments. In this study, the IVIVE approach provides a means of predicting bioaccumulation potential in humans, based on a limited number of physicochemical properties combined with *in vitro* measures of protein binding affinity and hepatic clearance. Finally, there is a need to develop sensitive throughput analytical techniques that allow a better determination of the kinetics parameters using *in vitro* experiments.

References

CEPA (1999): Canadian Environmental Protection Act. Government of Canada. *Canada Gazette Part* III.

ECHA (2008): Guidance on information requirements and chemical safety assessment. Chapter R.11: PBT Assessment. European Chemicals Agency, Helsinki, Finland.

ECHA (2012): Guidance on information requirements and chemical safety assessment. Chapter R.11: PBT Assessment (version 1.1). European Chemicals Agency, Helsinki, Finland. http://echa.europa.eu/documents/10162/13632/information_requirements_r11_en.pdf (*accessed 23 April 2013*).

Tonnelier, A., Coecke, S., Zaldivar, J.M. (2012): Screening of chemicals for human bioaccumulative potential with a physiologically based toxicokinetic model. *Arch. Toxicol.* 86: 393-403.

US EPA (1998): Proposed category for persistent, bioaccumulative, and toxic chemicals. Federal Registry, 53417-53423. United States Environmental Protection Agency, Washington, DC, USA.

4.10 Cross-Cluster Cooperation

4.10.1 The SEURAT-1 Roadmap

Mark Cronin, Barry Hardy, Elmar Heinzle, Jürgen Hescheler, Marc Peschanski, Catherine Verfaillie and the COACH Team

All **SEURAT-1** projects will individually or collaboratively contribute to the cluster level-objectives, which are: (i) to formulate and implement a research strategy based on generating and applying knowledge of modes-of-action; (ii) to develop highly innovative tools and methodology that can ultimately support regulatory safety assessment; (iii) to demonstrate proof-of-concept at multiple levels – theoretical, systems and application; and (iv) to provide the blueprint for expanding the applicability domains – chemical, toxicological and regulatory. Most of the **SEURAT-1** projects feed directly into the second objective (development of innovative tools and methodology), but they will also, through working groups (see below) or other coordinated cluster activities, contribute to demonstrating the proof-of-concept at multiple levels.

The three levels for proof-of-concept studies are

- to describe selected Modes-of-Action/Adverse Outcome Pathways to a sufficient extent so that they can be used as blueprints for system design;
- to demonstrate integrated systems for associating a chemical with a Mode-of-Action/Adverse Outcome Pathway category and for quantitative prediction;
- to use the information derived from predictive systems to support safety assessment processes and decisions.

Cross-cluster working groups were established (see the following section 4.10.2) in order to design the respective studies in relation to the three proof-of-concept levels. The identified proof-of-concepts (PoC) are regarded as cluster milestones, into which projects and working groups will feed. They are the backbone of the **SEURAT-1** roadmap, which was developed by COACH to provide a tool for monitoring project deliverables contributing to the **SEURAT-1** cluster objectives. Altogether, this roadmap will give an overview about cross-cluster interactions and cluster-level milestones, which are formulated to reach the cluster-level objectives.

The **SEURAT-1** timeline is illustrated in *Figure 4.61*, mapping out the milestones of the cluster. The 'Tools and Methodology catalogue' as one of the milestones, in the fifth year, will be the

collection of all tools and methodologies developed within **SEURAT-1**. Once completed, this collection will fulfill the second cluster-level objective (i.e., the development of highly innovative tools and methodology that can ultimately support regulatory safety assessment).

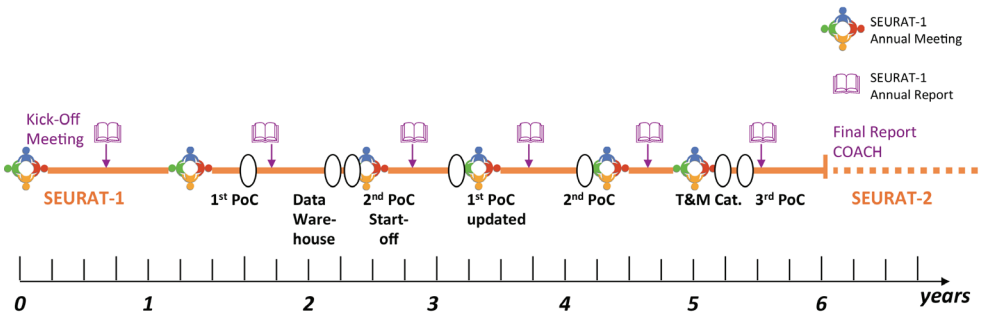


Figure 4.61 *The* ***SEURAT-1*** *timeline illustrating the timing of the proof of concept (PoC) at three conceptual levels and further milestones as the backbone for interactions between the* ***SEURAT-1*** *projects.*

At the beginning of **SEURAT-1** the deliverables from all projects were collected and compiled in a Gantt chart. This tool proved difficult to use as the deliverables were too numerous and detailed to give any useful overview. In addition, the Description of Work (DoW) of each project had not been developed in close collaboration with the other projects. It was therefore suggested to take a more top-down approach, working from the cluster-level objectives towards the key deliverables, being the project deliverables identified as essential for achieving the cluster objectives or triggering cross-cluster interactions.

The **SEURAT-1** project coordinators were first asked to identify the major milestones of their project contributing to the **SEURAT-1** objectives (presented in the second **SEURAT-1** Annual Report) and thereafter they identified the key deliverables from the project DoW contributing to these milestones. Based on this, the projects were incorporated into the roadmap (*Figure 4.62*) and the key deliverables became the basis for the **SEURAT-1** monitoring table and roadmap.

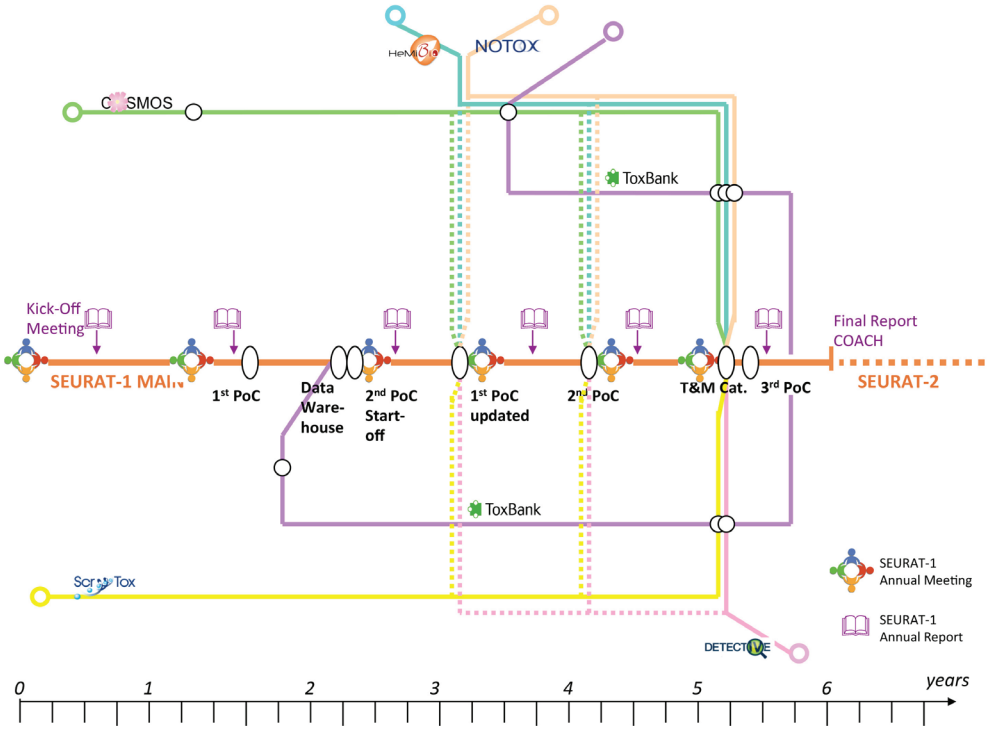


Figure 4.62 *The* ***SEURAT-1*** *roadmap illustrating the direct contributions from the projects to the cluster-level objectives.*

The roadmap has been created in such a way that it is possible to follow the timescale for the **SEURAT-1** cluster-level milestones in the main roadmap, while the timescale for each separate project or working group is elucidated in the segmented maps as described later. The dashed lines will be confirmed as the proof-of-concepts are developed within the cluster.

When **SEURAT-1** started it was recognised that additional deliverables, not covered by the **SEURAT-1** projects, would be needed to achieve the cluster objectives. Some of these were identified in an additional contract between the European Commission's Joint Research Centre in Ispra (JRC) and Cosmetics Europe, and this is illustrated by an additional line in the following roadmap (*Figure 4.63*).

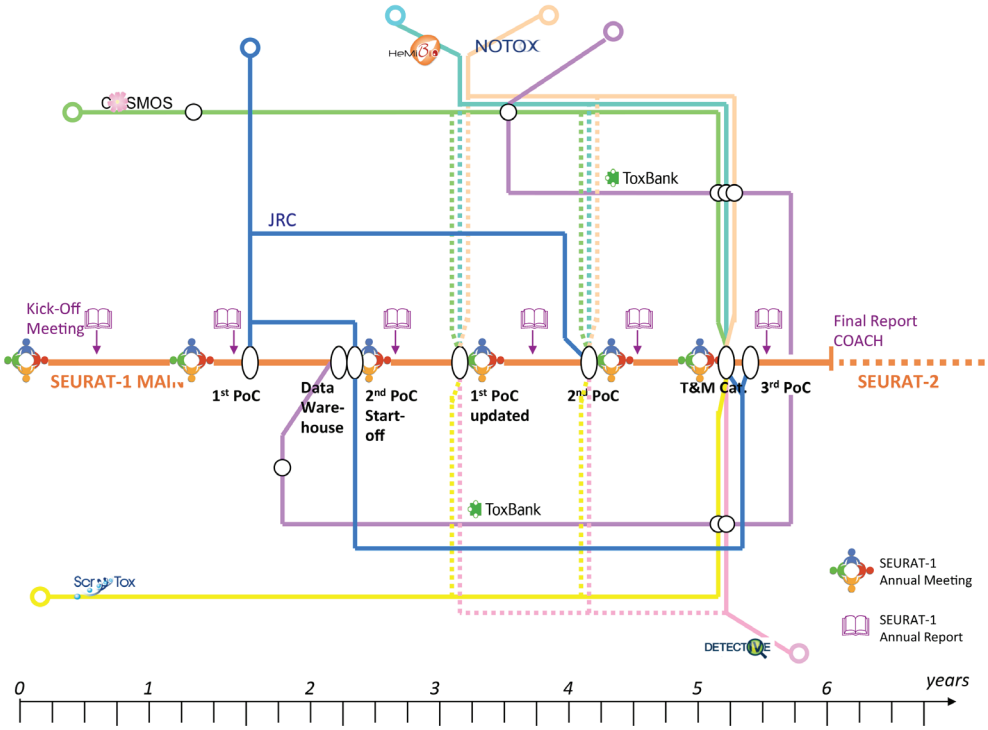


Figure 4.63 *The* ***SEURAT-1*** *roadmap illustrating the direct contributions from the projects and the JRC to the cluster-level objectives.*

Additionally, the **SEURAT-1** Working Groups were created to cover core-topic, 'horizontal' cross-cluster activities that reinforce the 'vertical' project pillars and are necessary for obtaining the common goals. The Working Groups are based on voluntary efforts from the partners to contributing to additional common output on a cluster level, but assist the partners in providing more relevant deliverables within the projects to meet the **SEURAT-1** objectives and benefit from progress and knowledge of other partners. Incorporating the Working Groups into the graphical representation completes the **SEURAT-1** roadmap (*Figure 4.64*).

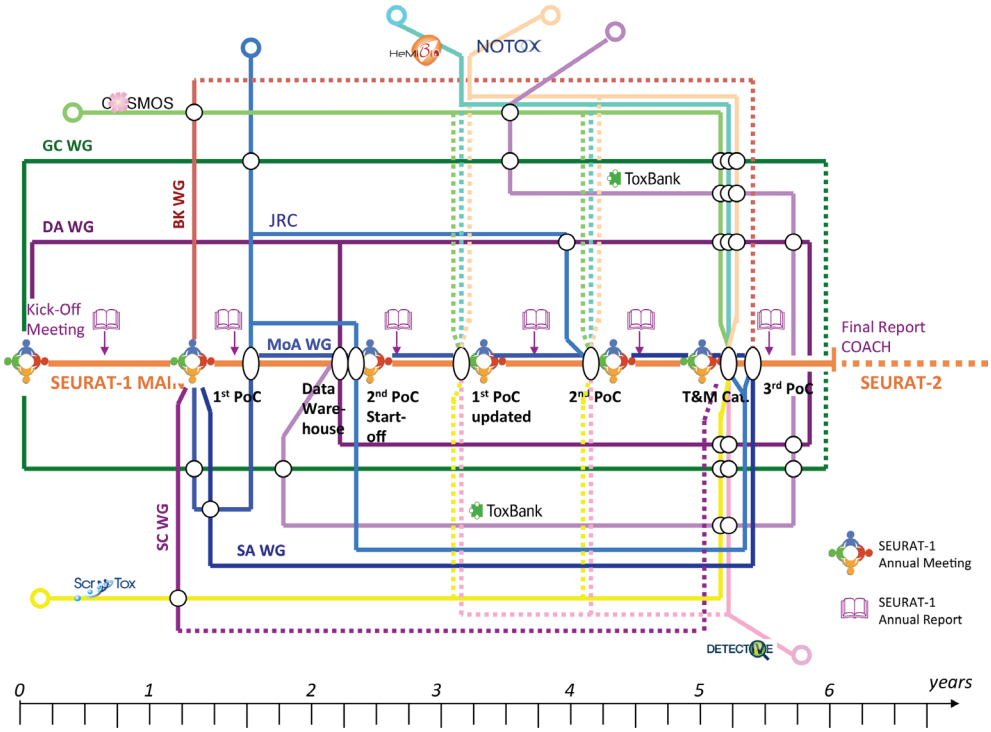


Figure 4.64 *The* ***SEURAT-1*** *roadmap illustrating the contributions from the projects, the JRC and the Working Groups to the cluster-level objectives.*

The Gold Compound (GC) and Data Analysis (DA) Working Groups had already started before the kick-off meeting. The GC WG in particular made an important contribution to meeting the cluster strategy, by selecting the chemicals to separately cover the main modes-of-action related to liver toxicity. The DA WG primarily supported ToxBank to develop the Data Warehouse, and is strongly supporting all partners in populating the warehouse with **SEURAT-1**-generated data. The Biokinetics (BK) and Stem Cell (SC) Working Groups started to develop a working programme at the third annual meeting. The Mode-of-Action (MoA) and Safety Assessment (SA) Working Group strongly progressed towards their individual goals in the second year of cluster activities, thereby contributing to the first Proof-of-Concept (MoA WG, see section 4.10.5) and third Proof-of-Concept (SA WG, see section 4.10.8), respectively.

The coordinators were also requested to provide information about which key deliverables from other projects were essential for their own project. The outcome is visualised in the separate project lines below (*Figures 4.65–4.70*). Only the filled circles and arrows illustrate required links between projects while the open ones can rather be regarded as 'nice to have'. Considering the Proof-of-Concept focus within **SEURAT-1** starting from the third annual meeting, especially taking into account the cluster-wide activity on defining case studies for

the second level Proof-of-Concept (development of integrated test systems based on mode-of-action knowledge) to be implemented during the next two years, it is assumed that additional links in-between projects will be identified.

The main roadmap, the separate roadmap lines and the progress-monitoring table, which is the basis for all the roadmaps, are all updated every six months, and then presented to and discussed by the Scientific Expert Panel.

SCR&Tox: The selected *SCR&Tox* key contractual deliverables, contributing to the cluster-level objectives, are given in the following *Table 4.7*. The respective, project-specific roadmap is illustrated in *Figure 4.65*.

Table 4.7 *Key deliverables of SCR&Tox contributing to the cluster level objectives.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones	Comments
D1.4	Production of ready-to-use cells for toxicity testing	High-throughput implementation of the whole assay
D2.1	Profiling and functional characterisation of test cells	Characterised cell models
D2.3	Production of optimised ready-to-use cells for toxicity testing	Optimised *in vitro* systems for toxicity testing
D3.1	Profiling and functional characterisation of cells with reference to the selected toxicity pathway and toxicants	High-throughput implementation of the whole assay
D3.2	Assay development of the selected toxicity test	High-throughput implementation of the whole assay
D3.3	Validation of the selected toxicity test	High-throughput implementation of the whole assay
D4.1	Establishment of standard operating procedure for industrial-scale	Industrial prototype
D4.2	Transfer of the selected toxicity test to the industrial-scale platforms	Industrial prototype
D4.3	Proof-of-Concept of the selected toxicity test implemented on industrial-scale platforms	Industrial prototype

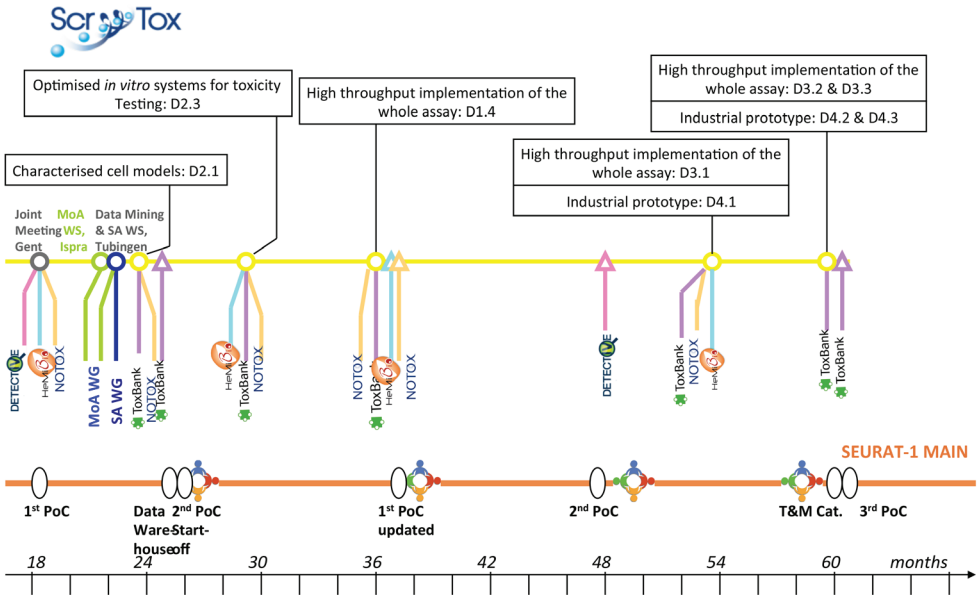


Figure 4.65 *The SCR&Tox roadmap illustrating the interactions between SCR&Tox, the other* ***SEURAT-1*** *projects and the Working Groups for achieving the cluster-level objectives.*

HeMiBio: A list of contractual deliverables, contributing to the cluster level, was compiled and is given in *Table 4.8*.

Table 4.8 *Key deliverables of HeMiBio contributing to the cluster level objectives.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones	Comments
D1.3	ZFN-mediated gene tagging in stem cell progeny	Completed for hepatocytes; pending for HSC and LSEC
D2.1	ZFN-mediated insertion of reporter cassettes in stem cell progeny	Master cell lines created; RCME effective
D2.2	Development of sensors	Functioning currently: K+; O2; glucose; pH; ammonium/urea; ALT/ glutamate
D3.1	Development of 2D patterning bioreactor	Mostly done
D4.2	Development and initial assessment of final 3D bioreactor	
D4.3	Assess function of hepatocytes, HSEC and HSC in 'final' 3D bioreactor	
D5.2	Optimisation and application of functional tests for liver function and toxicity during bioreactor development	Initial assessment of toxicity using Upcyte cells in 2D bioreactor done

The *HeMiBio* roadmap regarding contributions to the cluster-level objectives is illustrated in *Figure 4.66*. As it relates to D1.3 and D2.1 as well as D3.1, D4.2 and D4.3 *HeMiBio* organised a meeting with *SCR&Tox*, DETECTIVE and NOTOX in Gent in June 2012, to discuss possible collaboration/interactions in cell engineering as well as bioreactor engineering between the different consortia (a workshop summary report is given in section 4.10.9). Exchanges were continued in Lisbon in March 2013 during the **SEURAT-1** Annual Meeting, and a follow-up joint meeting on 'Bioreactors and Cell Engineering' is planned in September 2013.

As it relates to deliverables D2.1 and D2.2, *HeMiBio* is: (i) requesting input from ToxBank and other **SEURAT-1** partners related to levels of cosmetics and other toxic components that cause fibrosis, cholestasis and steatosis, and the metabolites of these molecules that can be measured in the cell and their surroundings, so that *HeMiBio* can build electrodes that can measure these components in the dose range both inside and outside the cell; (ii) asking for precise information regarding the extracellular levels we can expect of a number of components, including ALT, LDH, glutamate, lactate, glucose and others, because there is a need to optimise electrodes to be integrated in the bioreactor; (iii) looking for information about 'biomarkers' to allow the incorporation of molecular sensors in different cell components in the bioreactor. *HeMiBio* proposed a list of biomarkers at the end of February 2013 as a basis for discussion.

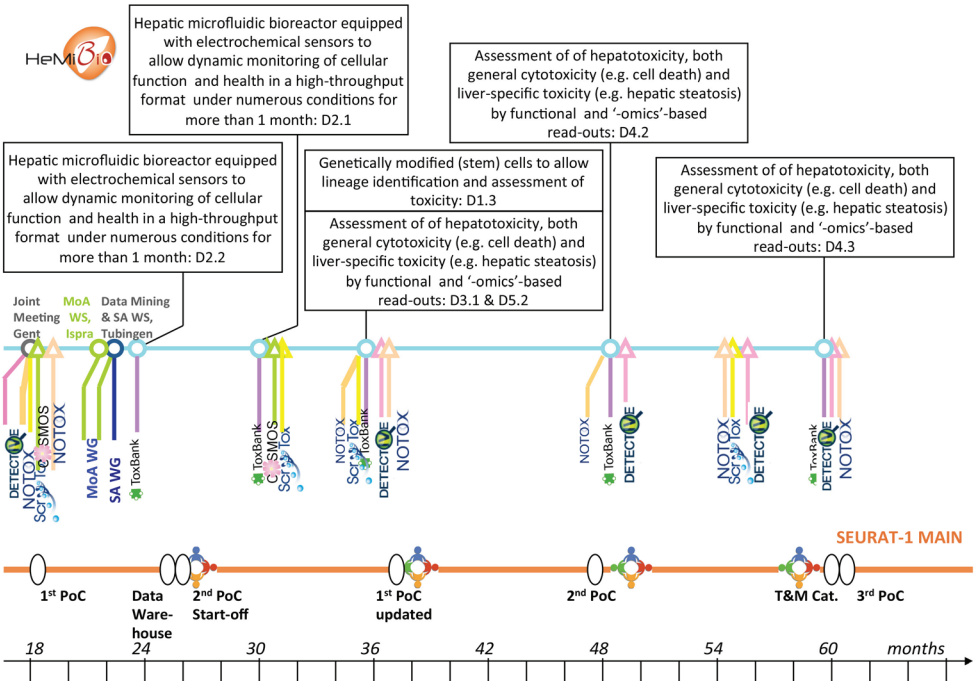


Figure 4.66 *The HeMiBio roadmap illustrating the interactions between HeMiBio, the other* ***SEURAT-1*** *projects and the Working Groups for achieving the cluster-level objectives.*

DETECTIVE: The following list of key deliverables for contribution to the cluster-level objectives has been identified (*Table 4.9*).

Table 4.9 *Key deliverables of DETECTIVE contributing to the cluster level objectives.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones
D6.1	Cardiac toxicity of compounds as determined by combined MEA/impedance measurements
D7.1	Cardiac and hepatic toxicity (using impedance measurements)
D8.1	Report on assays in cardiomyocytes and hepatotocytes (using high-throughput imaging)
D8.2	Report on imaging protocols for the identification of specific mechanisms leading to hepatoxicity
D9.2	Recommendations for cell type-specific functional assays in high throughput *in vitro* screening
D10.1	Complete atlas of gene expression
D12.1	List of candidate protein biomarkers from differential proteomic experiments
D12.2	Development of candidate biomarkers and prototype test formats, validation of reversibility effects
D12.3	Validation study of prototype test
D13.1	Identification of candidate metabolic biomarkers
D13.2	Definition of optimised protocols for detection of best candidate metabolic biomarkers
D15.1	Final report on the selected functional and '-omics' readouts as *in vitro* biomarkers

The DETECTIVE roadmap regarding contributions to the cluster-level objectives is illustrated in *Figure 4.67*. The key deliverables listed above are important for the cluster as they will be an important brick in the overall **SEURAT-1** cluster objective of developing a new alternative approach to current toxicity testing methods, with predictive power at least as good as animal-based methods. The DETECTIVE strategy will be validated using the cell systems developed by other **SEURAT-1** projects (particularly *SCR&Tox* and *HeMiBio*). This will be supported by the evaluation report, which will be made available to the other projects.

The data made available through the listed key deliverables could be helpful for the projects *HeMiBio* and COSMOS, and vice versa. Through collaborations with these projects DETECTIVE could, for example, help to identify qualitative and predictive biomarkers (e.g., for steatosis) and define the mode-of-action of specific compounds. ToxBank will be involved at every key deliverable for mutual management of all available data.

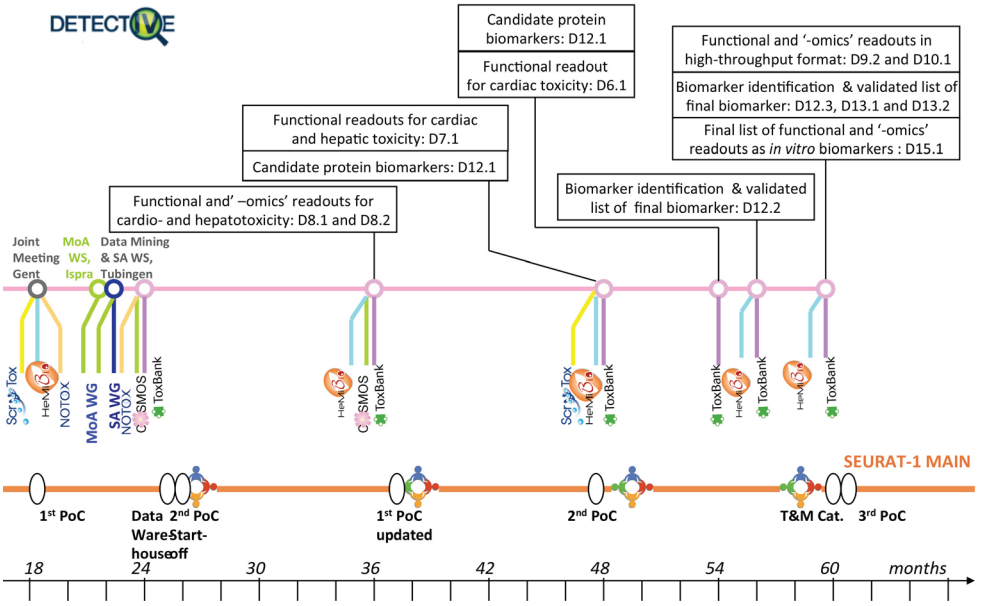


Figure 4.67 *The DETECTIVE roadmap illustrating the interactions between DETECTIVE, the other* ***SEURAT-1*** *projects and the Working Groups for achieving the cluster-level objectives.*

COSMOS: Contributions were divided into four different areas: (i) toxicity database and cosmetic materials inventory; (ii) TTC approach for cosmetic ingredients; (iii) AOP-derived models for organ-level toxicity; and (iv) biokinetics models to assist in *in vitro* concentration to *in vivo* dose extrapolation. The key deliverables are given separately for these four different areas.

Toxicity database and cosmetic materials inventory: The COSMOS database of toxicological information for cosmetic ingredients (and beyond), including the COSMOS Cosmetics Inventory, provides the backbone of the development of alternative models and forms a robust platform to collect, organise and mine highly curated and quality assured toxicity *in vivo* and *in vitro* data. It will contribute for the development of alternatives in the other **SEURAT-1** projects by access to high quality data. The COSMOS database will be made available in 2013 and also be linked to the ToxBank data warehouse. The related key deliverables are listed in the following *Table 4.10*.

Table 4.10 *Key deliverables of COSMOS in the area of toxicity databases and the COSMOS Cosmetics Inventory.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones
D1.7	Data summarisation algorithm
D1.8	Database format. Scheme for toxicology data and model representation
D1.9	Preliminary curated databases of toxicity data and information
D1.10	Assignment of quality scores and summarised values to preliminary databases
D1.11	Completed databases of curated and quality controlled information
D6.4	Preliminary databases on the project website
D6.5	Completed databases available on the project website

Updated TTC approach for cosmetic ingredients: COSMOS is developing Threshold of Toxicological Concern (TTC) approaches better suited to classes of cosmetic ingredients, in order to support efficient, ultimately regulatory, safety assessment. The TTC approaches have updated current knowledge and will be supported by the capability to build them on mechanistic knowledge, specifically by implementing the AOP approach to provide the link from chemistry to toxicity pathways. Related key deliverables are listed in *Table 4.11*.

Table 4.11 *Key deliverables of COSMOS in the area of Threshold of Toxicological Concern approaches for cosmetic ingredients.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones
D2.2	TTC approach adapted to cosmetics
D2.3	New TTC datasets
D2.4	TTC approach extended to target organ (repeated dose) toxicity for cosmetics
D2.7	Final TTC software tool delivery

AOP-derived models for organ-level toxicity: COSMOS is providing a number of innovative computational tools for organ-level toxicity prediction, which are being built around the COSMOS database and Cosmetics Inventory. In particular, chemical categories have been developed from knowledge derived from AOPs. These will be extended into more quantitative approaches to toxic potency, e.g. (Q)SARs, and will be refined to incorporate kinetic and

metabolic studies to permit quantitative interpretation of results in terms of consumer risk. The AOP approach provides a transparent link from chemistry to toxicological effect. COSMOS supports the development and promotion of AOPs, in particular by organising the chemistry involved in the process, e.g. through significant involvement in the **SEURAT-1** Mode-of-Action (MoA) Working Group. COSMOS thus contributes to the **SEURAT-1** objective of generating and applying MoA knowledge. Related key deliverables are given in the following *Table 4.12*.

Table 4.12 *Key deliverables of COSMOS in the area of AOP-derived models for organ-level toxicity.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones
D3.3	Grouping and read-across applied on the chemical space of the COSMOS Cosmetics Inventory
D3.6	New QSAR regression and classification models
D6.4	Preliminary models and workflows on the project website
D3.8	Methods and QSAR/computational models delivered as software tools
D6.5	Completed models and workflows available on the project website

Biokinetics models to assist in IVIVE: Models for toxicodynamics and toxicokinetics are being developed within COSMOS; these will extend capabilities for *in vitro–in vivo* extrapolation (IVIVE), allowing for better application of results from cell-based assays to perform human safety assessment. Research includes kinetics modelling (e.g. through physiologically based pharmacokinetic (PBPK) models); a better understanding of the effect of the test system (e.g. sorption) and chemical properties (e.g. volatility, stability) and how they relate to extrapolation; and modelling and prediction of metabolism. These models can be used to determine the internal exposure (dose at target organ level) necessary to elicit the effect. COSMOS will thus help to identify highly targeted assays within **SEURAT-1** that could be developed and used to provide evidence to support the **SEURAT-1** knowledge of pathways, also through coordinating efforts in the COSMOS co-lead Biokinetics Working Group. Related key deliverables are listed in *Table 4.13*.

Table 4.13 *Key deliverables of COSMOS in the area of biokinetics models.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones
D4.4	Modelling cell based assays using a Dynamic Energy Budget (DEB) approach
D4.2	Calibration of PBPK models based on *in vitro* data sets
D4.3	KNIME module for reactivity and metabolism prediction at cell line and at organ level
D3.5	Preliminary analysis of the possible incorporation of kinetic and metabolic studies
D4.5	Internal concentration in *in vitro* experiments and values as support in the implementation of the TTC approach
D6.4	Preliminary models on the project website
D4.6	Coupling molecular-cellular-organ and organisms levels for a selected case study
D4.7	Modelling simulation and validation of molecular metabolic and control networks for selected cell lines. Mode of interaction approach to toxicity
D4.8	Extrapolating *in vitro* to target organ level *in vivo*
D4.10	Analysis of the multiscale modelling approach for the understanding of toxic effects in cosmetic ingredients
D6.5	Completed models available on the project website

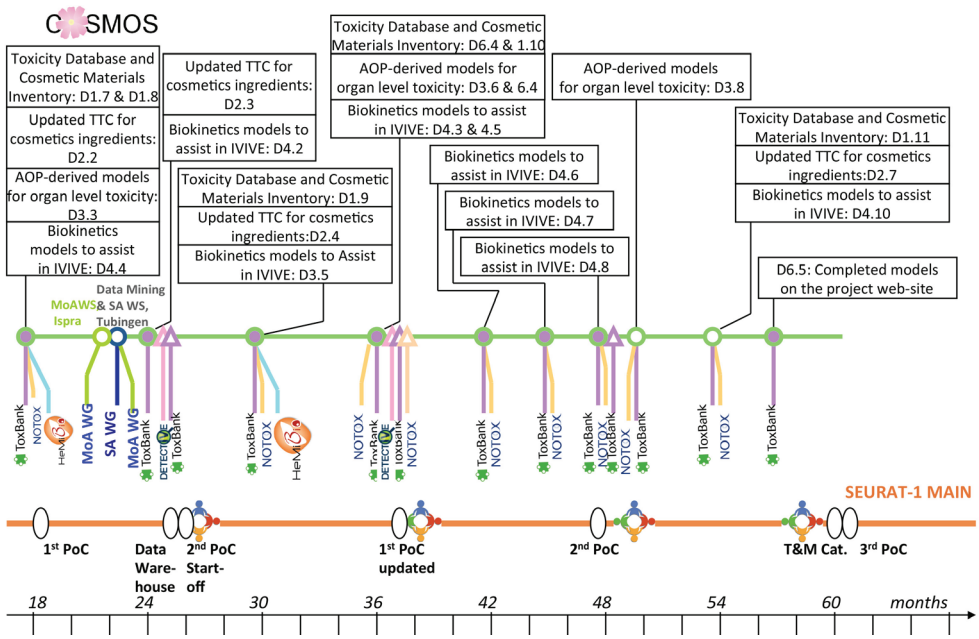


Figure 4.68 *The COSMOS roadmap illustrating the interactions between COSMOS, the other* ***SEURAT-1*** *projects and the Working Groups for achieving the cluster-level objectives.*

NOTOX: The selected NOTOX key contractual deliverables, contributing to the cluster-level objectives, are given in the following *Table 4.14*. The respective, project-specific roadmap is illustrated in *Figure 4.69*.

Table 4.14 *Key deliverables of NOTOX contributing to the cluster level objectives.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones
D1.3	Physiological data on long-term operation of membrane reactor using HepaRG
D1.4	Physiological data on long-term operation of HepaRG sandwich culture
D2.2	Quality test of MeDIP and ChIP on HepaRG and HepG2 cells, collection of data for reference map
D2.3	Flux and metabolome data from selected test compound
D2.4	Online visualisation tool for visualising hypothesis about the mode-of-action on the molecular level
D2.5	Validation of reference maps using 454 bisulphite sequencing for 40-50 amplicons
D2.6	Model of biological pathways affected by model compounds
D3.2	Prototype of the data analysis and visualisation algorithms
D3.3	3D reconstructions and tomographic averages with and without test compounds
D3.4	Integrated model of liver toxicity after long-term exposure to hepatotoxic compounds
D4.1	Prototype monolayer model
D4.3	Prototype sandwich model
D4.4	Prototype human organoid models
D4.5	Model for simulating long-term toxicity effects of test compounds
D4.6	Model for classifying response to toxin
D4.7	List of identified predictive endpoints

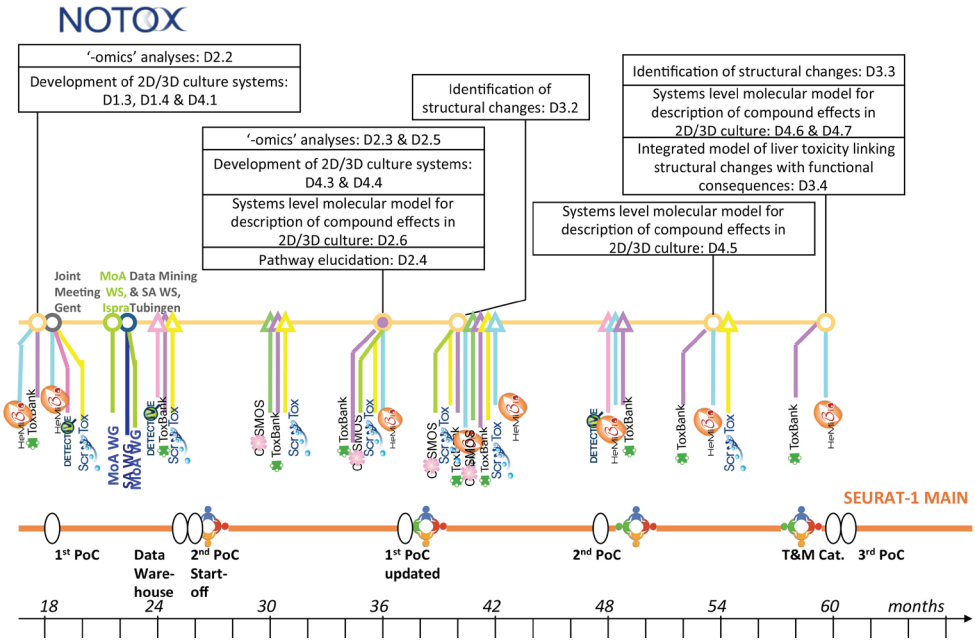


Figure 4.69 *The NOTOX roadmap illustrating the interactions between NOTOX, the other* ***SEURAT-1*** *projects and the Working Groups for achieving the cluster-level objectives.*

ToxBank: The selected ToxBank key contractual deliverables, contributing to the cluster-level objectives, are given in the following *Table 4.15*.

Table 4.15 *Key deliverables of ToxBank contributing to the cluster level objectives.*

Del. no.	Contractual deliverable identified as key deliverable to achieving the **SEURAT-1** major milestones
D1.2	Implementation of web interfaces, web services and data marts for Data Warehouse
D1.3	Incorporation of graphical user interfaces, data analysis, mining and model building analytics and tools
D1.4	Report on support and training services for ToxBank Warehouse users and enforcement and compliance with procedures for project-based ToxBank Data Warehouse data submission, curation and maintenance
D1.5	Final Report on development, implementation, performance, support and use of ToxBank Data Warehouse throughout the **SEURAT-1** Research Initiative
D2.2	ToxBank Gold Compound Database including high-quality data passing curation evaluation and acceptance criteria
D2.3	Procedures and analytical tools for selection of compounds for *in vitro* R&D programs, QSAR model building and training, integrated testing strategies, and model development and validation towards regulatory acceptance under REACH and the Cosmetics Directive
D2.4	Report on procedures compliance, support and training services for ToxBank Gold Compound Database users
D2.5	Final Report on development, implementation, performance, support and use of ToxBank Gold Compound Database throughout the **SEURAT-1** Research Initiative
D3.2	Entry of structures, physicochemical, stability and binding properties of test chemicals in Data Warehouse
D3.3	Analytical methods for unstable compounds
D4.3	Summary of existing European biobanks and biorepositories
D4.7	Inventory and map of European suppliers: materials, resources, facilities and standards
D4.9	Directory of suppliers of Materials for *in vitro* toxicology

These key ToxBank deliverables contributing to cluster objectives are distributed over five different areas: (i) the 'ToxBank Data Warehouse' for managing protocols and data supporting knowledge sharing and an integrated analysis at the cluster level (D1.2, D1.4, D1.5). The first versions of the warehouse were released for initial testing in 2012, and will be updated periodically throughout the duration of **SEURAT-1**; (ii) the 'ToxBank Gold Compound Information Resource' of reference compounds (D2.2, D2.3, D2.4, D2.5) supporting cluster-level testing of assays against a mode-of-action framework. The resource, currently implemented as a

wiki (www.toxbank.net), includes selection criteria, curated compound information, literature information on mechanism, chemical and physical properties, and availability of existing data sets including '-omics' data. This resource was initially released in 2011 and will be updated periodically throughout the duration of **SEURAT-1**; (iii) the 'ToxBank Repository of Test Chemicals' (D3.2, D3.3), including protocols for data quality characterisation and definition of criteria for the distribution, handling and stability of test chemicals to cluster participants; (iv) the 'ToxBank Biomaterials Information Resource' (D4.3, D4.7, D4.9), including information on biomaterials such as biological reagents and stem cell lines developed and used within the cluster, quality and regulatory criteria for establishment and dissemination of stem cell lines for toxicity testing, and information on existing European biobanks and biorepositories, including an evaluation process for supply of stem cell lines; and (v) the ToxBank procedures and analytical tools for integrated data analysis (D1.3) including selection of test compounds, *in vitro* tests and experimental factors, QSAR model building and training, '-omics' analysis, weight of evidence framework, and model development and validation.

The ToxBank roadmap towards the cluster-level objectives is illustrated in *Figure 4.70*.

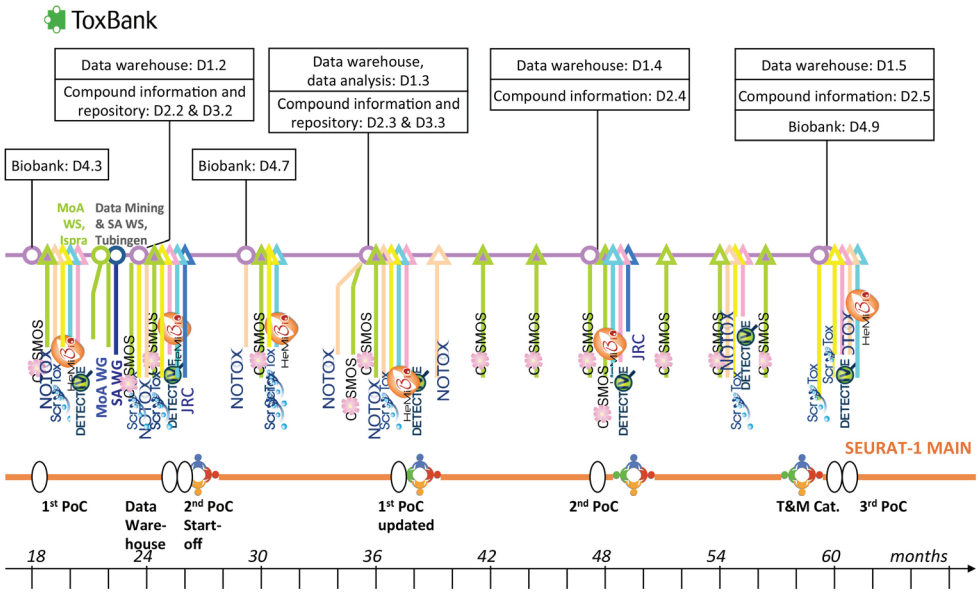


Figure 4.70 *The ToxBank roadmap illustrating the interactions between ToxBank, the other* ***SEURAT-1*** *projects and the Working Groups for achieving the cluster-level objectives.*

JRC: As mentioned earlier, a separate roadmap line was developed for the contributions from the European Commission's Joint Research Centre (JRC) to the cluster. In this case, all contractual deliverables are key for the cluster, as they were defined for exactly this purpose. The JRC roadmap is illustrated in *Figure 4.71*.

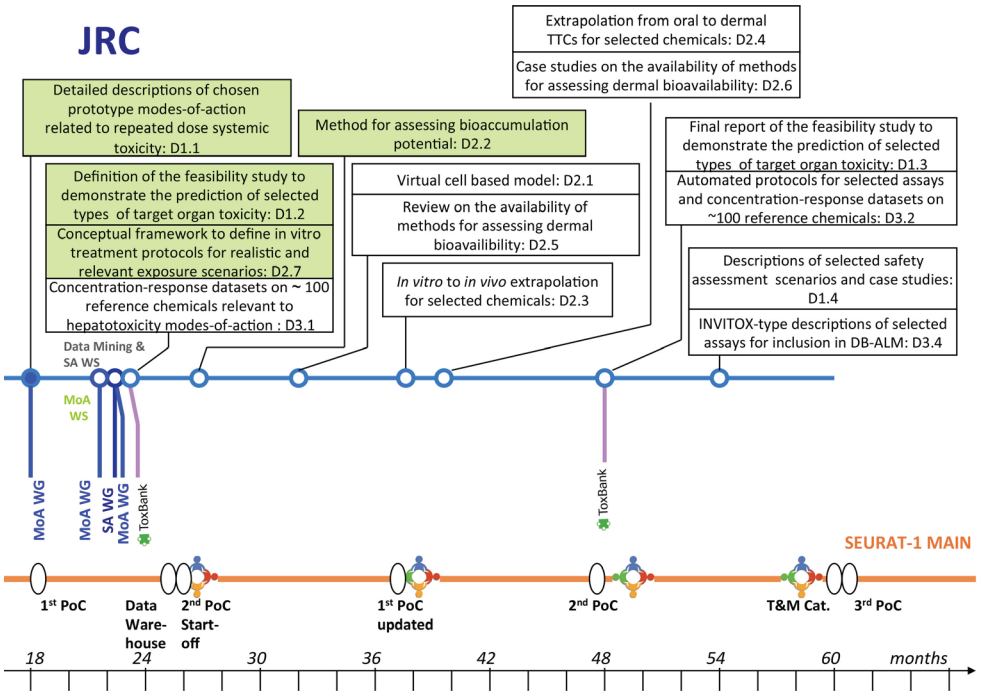


Figure 4.71 *The JRC roadmap outlining the JRC contributions to the cluster-level objectives.*

4.10.2 The Model of Cross-Cluster Working Groups

As briefly described in the previous chapter, Working Groups were created to facilitate the cross-cluster cooperation between projects and people. The overall motivation for establishing these cross-cluster working groups was to: (i) stimulate project interactions; (ii) assist the linkage of deliverables from different projects (in an effort to create the cluster-level roadmap); (iii) capture the knowledge spread over the more than 70 partners of the **SEURAT-1** Research Initiative. The challenge was to encourage collaborations not foreseen in the lists of deliverables of the individual projects and to find a way to broaden the reach of the **SEURAT-1** Research Initiative. It was therefore agreed by the Scientific Expert Panel of the **SEURAT-1** Research Initiative that a Working Group should have two aspects to its profile: one *Operational* aspect to deal with specific research questions and problems originating from project activities, and therefore finding common solutions on a cluster level, and a *Think Tank* aspect to encourage creativity and capture external expert views with the aim of achieving a large and multidisciplinary perspective.

A more detailed description about the establishment of the Working Groups, including Terms of References, is given in the second volume of the **SEURAT-1** Annual Report. The

following *Table 4.16* provides an overview about the existing Working Groups including short descriptions (more detailed working group reports are given in the following sections).

***Table 4.16 SEURAT-1** Working Groups in 2013.*

Working Group	Co-leaders	WG Description
Gold Compound	Jeff Wiseman (ToxBank) Paul Jennings (DETECTIVE)	The goal for the Gold Compound Working Group is to achieve consensus across the **SEURAT-1** Research Initiative on the criteria for selecting, accepting and using test substances in the development of alternative testing methods for repeated dose systemic toxicity. Cross-project members and additional external experts collaborate on the discussion of compound selection, mechanisms and assays. A criterion for the compound selection is a preference for previously well-studied compounds for which there is a good understanding of Mode-of-Action.
Data Analysis	Glenn Myatt (ToxBank) Annette Kopp-Schneider (DETECTIVE)	The Data Analysis Working Group discusses, on an ongoing basis, best practices, standards and common approaches for program data management and analysis, including topics such as vocabularies, protocols, ontologies, statistical analysis and integrated data analysis. The group also develops ideas and new approaches to data analysis required by emerging research activities carried out under the programme. The DAWG would also be suitable to host the discussions on the choice of biomarkers and approaches to the processing and analysis of associated '-omics' data.
Mode-of-Action	Mathieu Vinken (*HeMiBio* / DETECTIVE) Brigitte Landesmann (COACH)	The Mode-of-Action (MoA) Working Group assists in achieving the **SEURAT-1** objective to formulate and implement a research strategy based on generating and applying knowledge of MoAs. The MoA Working Group should identify known Modes-of-Action to support the data analysis and outcomes from different projects. It is suggested to use the Adverse Outcome Pathway (AOP) framework approach as a practical tool to organise MoA information and capture inter-relations in the cell by means of '-omics' and *in vitro* data including dose dependencies. A special focus is made trying to link Molecular Initial Events to possible adverse outcomes.
Biokinetics	Alexandre Pery (COSMOS) Emilio Benfenati (ToxBank)	The Biokinetics Working Group provides support to cluster activities in the paradigm shift from pure experimental approaches to a guided model-based approach. The Working Group will assist **SEURAT-1** projects to design *in vitro* and bioreactor models and experiments applied to those. To enable *in vitro* to *in vivo* extrapolation, the partners need to provide the working group with concentration measurements and effects data from the *in vitro* experiments. The efforts of the working group give strong support to achieving the **SEURAT-1** objective to develop highly innovative tools and methodology that can ultimately support regulatory safety assessment.

Stem Cells	Christian Pinset (*SCR&Tox*) Susanne Bremer (DETECTIVE/ *SCR&Tox*)	The aim of the Stem Cells Working Group is to standardise quality control issues of the cells used between the different partners and projects. Three cross-consortia cell model subgroups are identified: PSC lines (DETECTIVE, *SCR&Tox*), EBs (DETECTIVE, *SCR&Tox*) and Differentiated cell lines (*HeMiBio*, DETECTIVE, *SCR&Tox*). The Stem Cell Working Group with support from its subgroups will make it possible to evaluate the competences and robustness of the cell models used and also to ensure that results from different projects using the same cell models are comparable.
Safety Assessment	Andrew White (Unilever) Derek Knight (SEP)	The Safety Assessment Working Group will aim to bridge the gap between non-animal toxicity testing and the safety assessment decision-making needs. Future safety assessment approaches should based on the comprehensive knowledge of the Modes-of-Action and pathways leading to adverse effects in humans rather than animal testing. The working group will focus on applying the relevant information derived from the developing predictive systems across the projects to progress pragmatic solutions for addressing the safety decision needs. The group will examine what approaches are useful for building confidence and understanding the uncertainty within a mechanistic framework (for example, biokinetic modelling in combination with dose response analysis of *in vitro* results). As such the group will act as a facilitator to identify key gaps in current knowledge and data needs for the safety assessment decision, working across the regulatory and science space to ensure their generation, e.g. they will work with ToxBank to identify negatives that realistically help to define adaptive versus adverse effects.

4.10.3 Gold Compounds Working Group: Mechanism-based Selection of Reference Compounds for the Development of *in vitro* Toxicity Testing Methods

Jeffrey Wiseman[1]

4.10.3.1 Introduction and Objectives

The selection of standard reference compounds is a critical issue in any research programme that involves many research groups from different scientific disciplines and needs to be done according to the overarching goals or strategy of the programme. In the case of the **SEURAT-1** Research Initiative, the strategy and goals were outlined in the first Annual Report:

> 'The SEURAT strategy is to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure needed for safety assessment' (*Whelan & Schwarz, 2011*).

1. On behalf of the Gold Compounds Working Group

The core concept of how to select the appropriate reference compounds to meet these goals was extensively reported in the second volume of the Annual Report (*Benfenati et al., 2012; Wiseman, 2012*). In brief, the selection procedure was based on the following basic considerations: (i) extrapolations from well-studied reference compounds to a broader *chemical space* should be possible; (ii) *promiscuity*, that is, a lack of structural specificity in ligand binding, should be considered; (iii) the reference compounds should have well-known *modes-of-actions*; (iv) the reference compounds should be appropriate for studying *repeated dose toxicity*.

The selection of reference compounds is key to the success of a mode-of-action based approach and should be based on knowledge of different pathways predicted from both chemical and biological information. The starting point is indeed to select chemicals that have been extensively studied, i.e., those that are very well characterised with respect to their MoA profiles; this became the major task of the Gold Compounds Working Group. The selection started with addressing hepatotoxicity, but expanded over time to other organs of interest studied within the **SEURAT-1** Research Initiative. The objectives for the second year were:

- to complete the selection of hepatoxicity standards with inclusion of nuclear hormone receptor ligands;
- to complete the selection of nephrotoxicity standards;
- to advise the neurotoxicity team on the design of experiments and selection of reference compounds.

4.10.3.2 Gold Compound Selection Team

The compound selection strategy has evolved through consultation across the **SEURAT-1** Research Initiative. Each project team, the Scientific Expert Panel and Cosmetics Europe provided representatives at a kick-off meeting in Cascais, Portugal, in February, 2011. An advisory Gold Compound Working Group with 20 members was assembled from the attendees at the Cascais meeting, and an evaluation team of six scientists was assembled from the Scientific Expert Panel, industry and academic labs to evaluate specific compounds for acceptance. As a matter of process, it was agreed that compounds recommended as standards require unanimous agreement by the evaluation team and will be submitted to the working group for review and comment before being accepted as gold compound standards.

4.10.3.3 Compound Summary Table

The following *Table 4.17* provides an overview of the standard reference compounds ('Gold Compounds') of the **SEURAT-1** Research Initiative. With the exception of the most recently identified standards, detailed descriptions for these selected compounds, including extensive tables of properties, may be found on the ToxBank wiki (http://wiki.toxbank.net/wiki/Main_Page).

Table 4.17 *Summary information for reference standards.*

Hepatotoxins			
Toxicant	**Initiating Mechanism**	**Adverse Event of Interest**	**Wiki Table**
	Reactive Molecules		
Acetaminophen	Non-selective thiol reagent	Cytotoxicity	Yes
Iodoacetamide	Selective thiol reagent	Cytotoxicity	Yes
Allyl alcohol	Selective thiol reagent, energy source	Fibrosis	Yes
DMNQ	Redox cycling	Cytotoxicity	Yes
CCl_4	Free radical generator	Steatosis, fibrosis	Yes
Aflatoxin B1	Lysine reagent	Apoptosis	Yes
	Mitochondrial Disruption		
Oligomycin A	Inhibition of complex V	Cytotoxicity	Yes
Rotenone	Inhibition of complex I	Cytotoxicity	Yes
FCCP	Proton gradient uncoupler	Cytotoxicity	Yes
	Promiscuous Binding		
Valproic acid	Membrane disruption, inhibition of fatty acid beta-oxidation	Steatosis	Yes
Chlorpromazine	Membrane disruption	Cholestasis	Yes
Amiodarone	Phospholipid binding, membrane disruption, inhibition of fatty acid beta-oxidation	Phospholipidosis, steatosis	Yes
	Selective Binding		
Methotrexate	Antifolate	Fibrosis	Yes
Bosentan	BSEP inhibitor	Cholestasis	Yes
Dirlotapide	Microsomal triglyceride transport inhibitor	Steatosis	Yes
Fluoxetine	Phospholipid binding	Phospholipidosis	Yes
Hygromycin B	Ribosome inhibitor	Cytotoxicity	Yes
	Nuclear Hormone Receptor Ligads		
T0901317	Dual LXR-PXR agonist	Steatosis	Yes
Rifampicin	PXR agonist	Negative control, steatosis	Yes
WY14643	PPARα agonist	Lipid metabolism disruption, proliferation	No
β-Naphthoflavone	AhR agonist	Lipid metabolism disruption	No
Tamoxifen	ER modulator	Epigenetics	Yes
Nephrotoxins			
$KBrO_3$	Strong oxidizing agent	Cytotoxicity	Yes
Ochratoxin A	Cytoskeleton disruption	Epigenetics	Yes
Cardiotoxins			
Doxorubicin	Topoisomerase inhibitor, redox cycling	Repeated dose organ failure	Yes
Antimycin A	Mitochondrial disruption, ROS	Cytotoxicity	No
E4031	hERG antagonist	Torsade de Pointes	Yes

Carbachol	Cholinergic agonist	Cell phenotyping	Yes
Isoproterenol	Adrenergic agonist	Cell phenotyping	No
Nifedipine	L-type Ca channel antagonist	Cell phenotyping	No
Neurotoxins			
Naphthol AS-E phosphate	CREB inhibitor	Mechanistic standard	
Forskolin	CREB activator	Mechanistic standard	No
DAPT	Notch1 inhibitor	Mechanistic standard	No
Rapamycin	mTOR inhibitor	Mechanistic standard	No
GSK2334470	PDK1 inhibitor	Mechanistic standard	No
Akt1/2 inhibitor	AKT kinase nhibitor	Mechanistic standard	No
Nocodazole		Inhibition of neurite out-growth	No
U0126		Inhibition of neurite out-growth	No
Acrylamide		Inhibition of neurite out-growth	No
Propofol		Inhibition of synaptogen-esis	No
Lead(II) chloride		Inhibition of synaptogen-esis	No
Chlorpyrifos	Organophosphate acetylcholinesterase inhibitor	Affecting cAMP signal-ling (CREB)	No
Diazinon	Organophosphate acetylcholinesterase inhibitor	Affecting cAMP signal-ling (CREB)	No
Dieldrin		Affecting cAMP signal-ling (CREB)	No
Ni^{2+}		Affecting cAMP signal-ling (CREB)	No
Tributyltin (TBT)		Affecting cAMP signal-ling (CREB)	No
Trimethyltin (TMT)		Affecting cAMP signal-ling (CREB)	No
PCB 153		Affecting Notch signalling	No
PCB 180		Affecting Notch signalling	No
Glutamate		Affecting PDK1/Akt / mTOR signalling	No
Generic Negative Controls			
D-Mannitol	NA	NA	No

The selection of mechanism-based reference compounds for the **SEURAT-1** project is now complete, barring identification of new requirements by the project teams. In the case of such new requirements the working group will give updates in the future Annual Reports, otherwise this report can be considered as the final one.

References

Benfenati, E., Myatt, G., Wiseman, J., Hardy, B. (2012): ToxBank: Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology. In: Gocht, T., Schwarz, M. [Eds.]: Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity. Vol. 2: The Consolidation of the Research Strategy. Paris / France (self-publishing): p. 204-243.

Whelan, M., Schwarz, M. (2011): SEURAT: Vision, Research Strategy and Execution. In: Schwarz, M., Gocht, T. [Eds.]: Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity. Vol. 1: Launch of the European Research Initiative on Human Safety Assessment. Paris / France (self-publishing): p. 47-57.

Wiseman, J. (2012): Gold Compounds Working Group: Mechanism-Based Selection of Reference Compounds for Toxicity Testing Procedures. In: Gocht, T., Schwarz, M. [Eds.]: Towards the Replacement of *in vivo* Repeated Dose Systemic Toxicity. Vol. 2: The Consolidation of the Research Strategy. Paris / France (self-publishing): p. 278-281.

4.10.4 Data Analysis Working Group: Integrated Data Analysis

Barry Hardy, Annette Kopp-Schneider, Glenn J. Myatt[2]

4.10.4.1 Introduction and Objectives

The objective of the Data Analysis Working Group (DAWG) is to support the data analysis needs of the cluster, including data collection, integration, analysis, as well as experimental design. It provides a forum to discuss issues or problems within the cluster as well as with other academic and industrial groups. This group will discuss best practices, standards and common approaches including topics such as vocabularies, ontologies, statistical analysis, and integrated data analysis. The group will also develop ideas and new approaches to data analysis required by emerging research activities carried out under the programme, such as the choice of biomarkers and approaches to the processing and analysis of associated '-omics' data.

4.10.4.2 ToxBank Data Warehouse and Data Analysis Working Group

A critical infrastructure project, the ToxBank Data Warehouse, came online at the start of 2013 to support the data analysis needs across the cluster. It provides a web-accessible shared repository of know-how and experimental results to support the **SEURAT-1** Research Initiative. Results and protocols are uploaded from the research activities of the cluster partners as well as relevant data and protocols from other sources, such as public databases

2. On behalf of the Data Analysis Working Group

containing toxicogenomics data. The project is taking advantage of existing open standards, particularly the Investigation/Study/Assay (ISA) infrastructure (ISA-TAB) format and tools (*Sansone et al., 2012*). This universal data exchange format will ensure information about an experiment is being collected across the cluster in a consistent manner, that terms including experimental factors are mapped onto existing ontology terms, and enables the integration of data generated outside the cluster. Each step of the experiment is linked to a detailed protocol and the warehouse entries are linked to other resources including the ToxBank gold compounds and biomaterials wikis and the MoA wiki developed at the European Commissions' Joint Research Centre (JRC) in Ispra, Italy.

The short-term focus of the ToxBank data warehouse project is to support individual project teams as they upload protocols and investigation results to the warehouse. At the same time the warehouse will be extended to support the integrated data analysis needs of the **SEURAT-1** Research Initiative.

4.10.4.3 Activities

In 2012 the DAWG held a workshop in Lisbon where data analysis issues relevant to the **SEURAT-1** Research Initiative were discussed. The workshop reviewed a number of on-going activities and discussed their implications related to data analysis, including the JRC's OECD Harmonised Template 201 initiative for reporting information on adverse outcome pathways, the use of the selected standard reference compounds ('Gold Compounds'), the use of the ISA-TAB data exchange format, and a discussion around '-omics' data analysis workflow. The importance of using the data to both explain mechanisms and for prediction was highlighted along with the need to appropriately validate any prediction model and ensure that any resulting signals are meaningful. It was considered important to use both systems biology and data-driven (machine learning) approaches as well as to integrate with Threshold of Toxicological Conern (TTC) concepts, toxicity databases, and PBPK models. A number of topics were reviewed that needed further development including how to harmonise or integrate bioinformatics approaches across the cluster, how to perform a dose response analysis with '-omics' data, and how to integrate different types of '-omics' approaches (e.g. transcriptomics, proteomics, metabonomics, and so on). The need to access complementary assays results (e.g. links with the US research programmes ToxCast or Tox21), was highlighted as a priority for the group.

To advance issues highlighted at the workshop, a number of DAWG activities and webinars were initiated, including webinars on complementary data sources including the DrugMatrix™ toxicogenomics database (*NTP, 2013*) and the Tox21 project (*Kavlock et al., 2009*), as well as informational webinars on the ISA-TAB universal data exchange format. There was also a webinar outlining the experiences of the Predict-IV project (*Wilmes et al., 2013*) to better understand how different '-omics' technologies might be combined as part of an integrated data analysis.

4.10.4.4 SEURAT-1 Data Analysis Framework

An important focus for the DAWG activities over the next year is the development of a **SEURAT-1** data analysis framework that will be used to support the cluster. The initial focus of the framework will be to support the **SEURAT-1** proof-of-concept case studies. As a kick-off to this activity a workshop was held at the third **SEURAT-1** Annual Meeting (March 2013 in Lisbon) where stakeholders from across the cluster as well as advisors to the project presented brief summaries of issues that were considered important. This workshop covered safety assessment within industry, the use of weight of evidence within the regulatory context, incorporation of knowledge of chemical categories, exposure protocols, analysis of integrated '-omics' technologies, linking evidence to pathways, biokinetics and evidence integration, statistical analysis and uncertainty, and **SEURAT-1** proof-of-concept needs.

In addition to this workshop, additional requirements will be collected through visits to partners in the cluster involved in this meta-analysis of the data. At the same time, the DAWG will continue its webinar series and include discussions including the use of MoA ontologies in projects outside of the **SEURAT-1** Research Initiative as well as other topics supporting the development of a **SEURAT-1** data analysis framework. It is planned to complete this framework analysis document by summer 2013, with recommendations providing input into the ToxBank Data Warehouse development as well as methodologies and workflows to support the mode-of-action and industrial prototype case studies.

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4.10.5 Mode-of-Action Working Group: Capturing Mode-of-Action Knowledge

Brigitte Landesmann, Mathieu Vinken

4.10.5.1 Introduction and Objectives

Following the second **SEURAT-1** Annual Meeting in February 2012, the Mode-of-Action Working Group (MAWG) was launched to facilitate cross-cluster cooperation between projects and people and to assist in achieving the following **SEURAT-1** cluster-level objectives: (i) to formulate and implement a research strategy based on generating and applying knowledge of modes-of-action (MoA); and (ii) to demonstrate proof-of-concept at multiple levels from theory to application. The objectives in the second year were

- to identify what mechanistic information each project within the **SEURAT-1** Research Initiative can contribute to the development of the mode-of-action framework;
- to outline how this information can be used in experimental design;
- to establish methods for capturing information about molecular initiating events (MIEs) and further downstream effects allowing for the identification of key events within an adverse outcome pathway;
- to integrate these efforts into international activities in the field (such as the development of adverse outcome pathways by the OECD).

4.10.5.2 Activities

After the endorsement of the Terms of Reference for the working groups by the Scientific Expert Panel of the **SEURAT-1** Research Initiative, Steve Enoch and Brigitte Landesmann were nominated as co-chairs and the MAWG became operational in June 2012. Due to time constraints, Steve Enoch could no longer act as co-chair of the MAWG from January 2013 on and this task was taken over by Mathieu Vinken. The group has 25 participants involving members from all six **SEURAT-1** projects.

At the beginning of July 2012, interested people from all projects were invited to attend a first teleconference with the intention of (i) setting up collaborations within the working group; (ii) discussing the intended strategy; (iii) exploring the respective position within the framework as well as common interests between the projects; and (iv) establishing communication rules within the working group.

As part of the additional activities of the JRC under the additional contract with Cosmetics Europe (see above, section 4.10.1), a report with detailed descriptions of two prototype MoAs related to repeated dose systemic toxicity, i.e., MoA from protein alkylation to liver fibrosis and MoA from LXR activation to liver steatosis, was completed in July 2012 (*Landesmann et al., 2012*). The purpose of this report was to define and sufficiently describe an MoA in order to facilitate conducting a feasibility study in accordance with **SEURAT-1** objectives. These two MoA descriptions were used as a first reference for the planning of further experiments and case studies within **SEURAT-1**.

In October 2012, the MAWG, together with COACH, organised a workshop in Ispra, Italy, for describing MoAs in liver toxicity using adverse outcome pathways (AOPs), with the objectives of evaluating practical processes and tools for describing and reporting an MoA/AOP, and considering how such MoA/AOP knowledge can be applied practically within all **SEURAT-1** projects. External experts attended the workshop and gave their input into the **SEURAT-1** efforts of elaborating the strategy of using AOPs as the conceptual framework for the overall **SEURAT-1** strategy (see chapter 3, a report from this workshop is given in the following section 4.10.5.3). An action list was formulated and the MAWG was assigned to the task of identifying the MoAs that are relevant for **SEURAT-1** research objectives and subsequently collecting available knowledge on these MoAs. Based upon the results, the Safety Assessment Working Group (SAWG) will then identify MoAs of concern from the safety assessment perspective.

This was followed by a workshop, entitled 'Exploring existing databases for modes-of-action of repeated dose systemic toxicity', in November 2012 in Tübingen, Germany, organised again by the MAWG in collaboration with COACH. The goal of this workshop was to develop a concept for mining the extensive information hidden in gene expression databases from acute and subchronic toxicity studies for defining relevant toxicity pathways. Again, international experts were invited and it was agreed that gene expression analysis could help to identify underlying mechanisms to fill the knowledge gaps in adverse outcome pathways, i.e., to formulate hypotheses for key events as a basis for the development of toxicity testing methods (see workshop report in section 4.10.5.4). The Safety Assessment Working Group held a workshop at the same time (see section 4.10.8.2) allowing direct exchange between these two working groups in a joint session at the end of the separate workshops, as a starting point of discussing **SEURAT-1** case studies.

At the beginning of 2013, another teleconference was organised with colleagues from all **SEURAT-1** projects who were willing to contribute to the activity of harvesting MoA knowledge.

A lead person for each organ investigated within the **SEURAT-1** Research Initiative was selected and a short questionnaire was sent to all **SEURAT-1** collaborators in order to collect as much input as possible from whole project cluster. A list of relevant candidate MoAs for each of the target organs, i.e., liver, kidney, heart, skin and the central nervous system, resulted from this activity (see section 4.10.5.5).

Brigitte Landesmann and Mathieu Vinken attended the workshop 'Building shared experience to advance practical application of pathway-based toxicity: liver toxicity mode-of-action' organised by the Human Toxicology Project Consortium (HTPC) in Baltimore, USA, at the end of January 2013. During this workshop, information on the **SEURAT-1** Research Initiative was given and the two liver toxicity pathways (*Landesmann et al., 2012*) were presented and discussed. Contacts were established with external experts in the field and the preparation of additional MoAs describing liver toxicity, in particular drug-induced liver cholestasis, was anticipated.

Finally, in March 2013, the MAWG organised together with COACH a workshop in Ispra, Italy, with the aim of identifying AOPs for human neurotoxicity and to prioritising the most relevant ones for further development (see section 4.10.5.6).

Planned activities for the third year of the **SEURAT-1** Research Initiative are:

- to further refine the already elaborated MoA descriptions;
- to elaborate additional MoA descriptions (the MoA for cholestasis resulting from the inhibition of the bile salt export pump has already been elaborated and will be published soon; based on the results of the neurotoxicity workshop in March 2013, expert groups will address one or more neurotoxicity AOPs);
- to contribute to the development of an AOP knowledgebase in collaboration with ToxBank within **SEURAT-1** and externally with the OECD and US Environmental Protection Agency.

The Mode-of-Action Working Group was very active in this phase of the **SEURAT-1** Research Initiative and contributed a lot to the advancement of the overall research activity (see chapter 3). A separate roadmap was created to illustrate the relationship with the research projects and the contributions of this working group for achieving the cluster-level objectives (*Figure 4.72*).

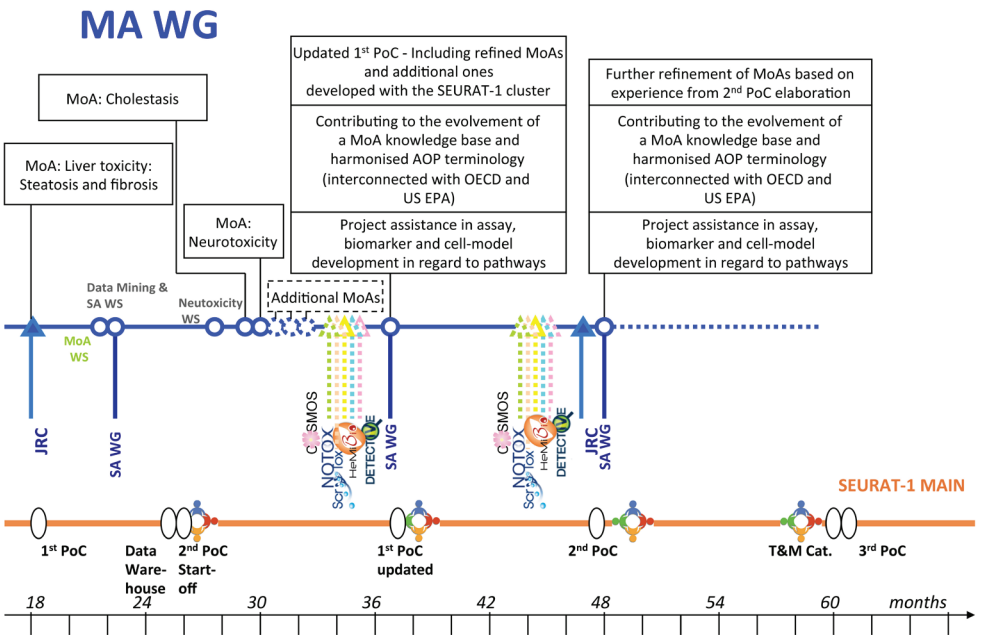

Figure 4.72 *The Mode-of-Action Working Group roadmap illustrating the interactions with the* ***SEURAT-1*** *projects for achieving the cluster-level objectives.*

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4.10.5.3 Describing Mode-of-Action in Liver Toxicity Using Adverse Outcome Pathways

Introduction

This **SEURAT-1** workshop on modes-of-action (MoA), organised by the **SEURAT-1** Mode-of-Action Working Group (MAWG) and COACH, was held from 24–25 October 2012 in Ispra, Italy. The outline of the workshop followed the profile of Working Group activities. The first

day was dedicated to the *Think Tank Aspect* and was intended to capture external experts' experiences and views on the current status of MoA/AOP/Toxicity-Pathway development with the emphasis being more on the building process itself, rather than on the actual content of the pathways. The second day dealt with the *Operational Aspect* and focused on specific AOP-related **SEURAT-1** research project activities and needs. A representative from each project was asked for a short presentation on the project's MoA/AOP-related activities and how they fit into the overall **SEURAT-1** framework.

The objectives of this expert workshop were to evaluate practical processes and tools for describing and reporting an MoA or AOP, based on worked examples and to consider how such MoA knowledge can be applied practically within **SEURAT-1**.

Day 1

Maurice Whelan opened the workshop and gave a short overview on the **SEURAT-1** Research Initiative. He reminded participants that, among the four strategic objectives of **SEURAT-1**, the third objective of 'proof of concept at multiple levels' is the most important; it represents the backbone of the whole project and its achievement will be one of the success criteria for **SEURAT-1**.

He mentioned the recommendations and identified problems from the previous year's workshop, then finally posed the main questions for day 1: (i) experiences so far and lessons for the future; (ii) the specific description of pathway characteristics; (iii) typical problems in the AOP development process; and (iv) the role of computational and experimental modelling.

'Lessons in Developing Adverse Outcome Pathways' (Terry Schultz): The discussion focused on the need to harmonise AOP terminology among the **SEURAT-1** partners. The differences between AOP and MoA and between key events and intermediate events should be clarified. The same scientific information is used in different approaches by OECD and WHO. The AOP approach of OECD focuses on how to use mechanistic information to build categories, develop integrated testing strategies, and inform the decisions for developing alternative test methods, while the MOA approach of WHO focuses on guidance on how to use mechanistic information to inform the risk assessment of individual chemicals.

Criteria are needed to define the level of detail that is necessary for an MoA description so that it can be considered accurate and robust. The MoA description process will vary depending on the purpose and on the availability of data. Minimal requirements for an AOP description are the definition of the two anchors, the Molecular Initiating Event (MIE) and the Adverse Outcome (AO), while the space between them will continually be filled with growing knowledge on Intermediate Events (IEs); the more knowledge that is available the more robust and accurate the description will be.

'Building AOPs: Example from Reproductive Toxicity' (George Daston): George Daston stressed the importance of identifying the molecular initiating event (MIE); further linking to advers

outcomes requires considerable hypothesis testing, which could be aided by simulations. Chemical ontology can aid in assigning chemicals to groups with the same putative MoA, but promiscuous chemicals might have more than one molecular target and seemingly similar compounds might have different developmental outcomes. Gene expression is specific to an MoA and its analysis can provide useful data to inform MoA and AOP.

'Evolving Approaches to AOP Development: Observations from Sensitization to Thyroid Hormone Pathways' (Kate Willett): Kate Willett concluded that AOPs can be built in a number of different ways, using information from a wide range of biological organisations and for a number of different decision-making purposes. The probable best approach is the combination of all these. She listed essential elements, namely good definitions of MoAs and AOs (for the relevant specific use); the description should include the context, diagrams, documented QC of data, well-curated linkages, and consistent definitions, terms and formats. For use in RA quantitative dimensions are needed, and integrated databases and 'knowledge bases' will be helpful.

'Adverse Outcome Pathways for Mitochondrial Toxicity' (Kendall Wallace): Key message: There exist multiple MIEs leading to mitochondrial toxicity, each with distinct intermediate eventss and adverse outcome profiles and each requiring the development of unique AOPs.

Ken Wallace stressed that the meaning of mitochondrial toxicity needs to be clearly defined; e.g. should a chemical that inhibits fatty acid oxidation or the Krebs cycle be considered to cause mitochondrial toxicity even if it doesn't directly interact with the mitochondrial respiratory chain?

'Inhibitors' (both direct and indirect) of mitochondrial respiration have to be distinguished from 'uncouplers' of oxidative phosphorylation – although both inhibit ATP synthesis, they elicit different IEs and cause dramatically different effects on oxidative intermediary metabolism. Direct inhibitors may act at different complexes of the electron transport chain and cause different effects, e.g. inhibitors of complex I produce less ROS than inhibitors of complex IV, while indirect inhibitors do not directly interact with complexes of the electron transport chain and may actually decrease ROS production. Both inhibitors and uncouplers, though starting with different MIEs, cause acute toxicity through inhibition of aerobic ATP synthesis and diverge again to differential metabolic profiles and disease phenotypes. There are multiple and distinct mitochondrial AOPs, depending on specific MIEs, with each requiring an independent development.

'Building Nuclear Receptor Mediated Liver Cancer AOPs' (Chris Corton): The presentation of Chris Corton brought the discussion to the need for standardised computational tools for finding and organising relevant information for a proposed MoA and for evaluating the weight of evidence. The participants agreed that computational tools should be used to support the MoA description process, though a standardised way for doing this still has to be defined. Genetic data might help to better describe an MoA. How much information is enough?

‘Building Pathways for *in vitro* to *in vivo* Extrapolation in the Virtual Liver Project’ (Imran Shah): Imran Shah gave a practical example of how computational tools might be used to connect data from different sources (such as chemical structure information, *in vitro* tests, metabolic pathway, text mining, etc.) in order to generate an MoA. The graphical display of the connection between an MIE and AO is problematic. Simply linking the different events of the AOP is not sufficient, since we also need to consider its kinetics, homeostasis and dynamics to be able to capture its complexity. We lack a symbolic language to demonstrate the functional and dynamic relationships between actors and events within an AOP.

‘Towards Adverse Outcome Pathways in Chronic Toxicity Modelling: Some Sobering Thoughts Arising from this Year Noble Prize in Physiology and/or Medicine’ (Ian Cotgreave): Ian Cotgreave highlighted some of the problems arising from new stem cell technologies that do not necessarily equally maintain MoAs. Stem cell-derived differentiated cells may not operate particular toxicity pathways equally to primary cells or traditional cell lines. Differentiated cells of similar phenotype, but derived from different stem cell origins (hESC and iPSC), may exhibit differences in sensitivity to MoAs of toxins.

‘The Crux of AOP Building – a practical example’ (Marina Goumenou): Marina Goumenou illustrated the multi-step approach following the OECD template for building an AOP and addressed the various difficulties of each step.

1. Understand what an MoA is about;

2. Establish the molecular initiating event and the adverse outcome you are interested in;

3. Specify the purpose of the MoA building and consequently the required detail;

4. Understand the physiology of the system;

5. Build the MoA searching for intermediate events;

6. Evaluate the MoA.

In the ensuing discussion it was repeated that an integrated approach using all the available tools (computation, databases, technical methods, etc.) is necessary for the detailed description of a specific MoA that captures all complexities. Further, the need for improved communication and knowledge-sharing between **SEURAT-1** partners was expressed. More importance should be given to the evaluation of the robustness and accuracy of an MoA. It was suggested to use a crowd-sourcing model for the description of a specific AOP with the **SEURAT-1** experts as peer reviewers and ToxBank serving as knowledge base. The reporting format could follow the OECD guideline.

'CREB: a pathway to grab neurotoxicity MoA' (Francesca Pistollato): Key message: As CREB-regulated downstream events are key to neuronal survival, CREB might be a relevant pathway to grab neurotox-related MoA.

Francesca Pistollato presented a well-described neuronal-related pathway, CREB (the cyclic-AMP response element binding protein), known to be involved in several neuronal processes, such as: neuronal survival; long-term synaptic plasticity; neuronal excitability; neurite outgrowth; adult SVZ (subventricular zone) neurogenesis; long-term potentiation; and drug addiction. Given the relevance of the CREB pathway in neurotoxicity and its upregulation in the pluripotent stem cell models (hESCs and hiPSCs) used at the JRC, it has been proposed as a candidate pathway for the design of a mechanistically driven neurotoxicity testing. During the presentation the selection process of the necessary elements for a toxicity pathway was shown. Ongoing experiments will address the relevance of CREB in chemical-induced neurotoxicity.

'Stem Cell-Derived Systems for Novel *in vitro* Systems Predicting Drug Hepatotoxicity; Experience from the SCR&Tox Project' (Magnus Ingelman-Sundberg): Key mesaage: The quality and usefulness of hepatocytes from stem cells is donor dependent and can be improved if the last steps in the differentiation programme are done in 3D cultures. Mesenchymal stem cells might provide a useful tool for identifying mechanisms and biomarkers for simvastatin toxicity. Hepatocytes derived from stem cells might be useful for screening of genotoxic versus non-genotoxic carcinogens and for evaluation of drug-induced steatosis.

Magnus Ingelman-Sundberg talked about the *SCR&Tox* project, which aims to provide proof of concept on the use of pluripotent stem cell lines for identifying 'toxicity pathways', i.e. key signaling pathways, the perturbations of which result in adverse health effects, and for setting up assays for assessing risks to trigger those pathways. This is be carried out in parallel in five main target organs for drug and cosmetic toxicity; namely the liver, heart, epidermis, nervous system and musculoskeletal system. He summarised results from, in particular, Cellectis, Karolinska Institutet and INSERM. He talked about mesenchymal stem cells as targets for simvastatin-induced toxicity and the identification of biomarkers for toxicity from these cells, efforts made to make differentiated hepatocytes from stem cells, and the use of 3D hollow fibre bioreactors for more efficient formation of stem cell-derived hepatocytes and for usage in predictions of drug hepatotoxicity.

From the discussion it was concluded that it is important to fully understand the MoA for tailoring an *in vitro* assay to capture a specific MoA. It is neither feasible nor desirable to mimic the *in vivo* situation as a whole. We need to develop an *in vitro* test expressing only the key characteristics required to capture an MoA without trying to reproduce the whole pathophysiology of a target organ *in vitro*.

Discussion (from the initially posed questions)

1. What can we learn from different experiences with MoA/AOP/Toxicity-Pathway development?

> ➠ The description of a pathway has to be specific. The events along an AOP might be converging and diverging, resulting in a kind of bottleneck on the way (different MIEs may lead to a same IE – e.g. Mitotox – and later diverge to various AOs).
>
> ➠ AOP description is dependent on the purpose, and thus the level of focus must be adequate to this purpose. However, the two anchors (MIE and AO) must always be well defined.
>
> ➠ An AOP will become more robust and precise as knowledge increases and the gaps between the two anchors can be successively filled.
>
> ➠ Test systems should be designed knowledge and not empirically driven, with a purpose clearly defined beforehand. The underlying mechanisms/modes-of-action have to be understood.
>
> ➠ Elaborated AOPs should be clearly reported, communicated and shared within the community.
>
> ➠ One proposed strategy for sharing an AOP and capturing its complexity was the software programming approach: define the scope, start with a simple design, test it and then eventually further expand and elaborate it. The concept of starting with simple things first is opposed to the view that the whole complexity has to be captured before simplification can be started.

2. How can we describe the characteristics, traits or attributes of various types/flavours of AOP? This was discussed together with the following question:

3. Can we identify and describe the typical problems encountered during the AOP development process and how can they be tackled?

> ➠ The various presented examples show different ways to describe the process, but all are facing similar problems.
>
> ➠ All presented AOPs were qualitative with identified actors and events; nevertheless, they are not fit for regulatory purpose yet, because quantitative descriptors such as dose-response data for MIE, threshold values, temporal concordance, etc. are missing. A detailed quantitative assessment for each step probably requires computational tools.
>
> ➠ There is a need for a common language, a common terminology. For example, there are various understandings of a key event; it might be conceived

either as any intermediate event, as a measurable intermediate event or as an intersection of different pathways. Does it need to lead irreversibly to adversity, or how should reversibility otherwise be weighed?

➠ AOPs are built around "favourite" key events; they need to become more objective.

➠ There was a debate on linearity: some of the participants considered that following the OECD template for the description of an AOP is a linear process, but this is too simplistic and therefore unable to capture the complexity of biological processes. Following this comment it was discussed that actually it is made to look linear following sequential levels of biological organisation, but the mechanistic description behind is not linear and depends on the level of observation. The AOP concept dictates linearity to simplify and visualise complexity, but we need to distinguish between static linearity, which describes a fixed one-to-one relationship focusing only on the qualitative aspect, and dynamic linearity, which also considers a quantitative relationship between the individual actors and events along the pathway.

➠ It is difficult to communicate dynamic and complex processes and we need descriptive terms that help describe the dynamic relationship between actors and events. We should aim for a multi-dimensional description with several matrices (biology, time, dose, events).

➠ Any kind of 'master switch' would be powerful if identified and incorporated in AOPs.

➠ Genomics can be used as a tool for pathway analysis that is distinctive between groups. Gene expression analysis might help to identify the associated biology and to fill the gap between MIE and AO. It is an underutilised tool.

➠ It is important not to work in isolation, but to create interoperable data and to interlink with as many tools as possible. Attractive tools and useful outputs will be incentives to make people collaborate. It was agreed that a wiki-like tool could be a good starting point for a standardised description of events. Once an AOP is described, it will be put into this tool to be judged and evaluated by the **SEURAT-1** community and possibly implemented.

➠ Relevant AOPs should be selected under the risk assessment aspect and those with regulatory interest should be prioritised; this task would preferably be done by the safety assessment working group.

4. How can we use computational and experimental modelling to help develop AOPs?

➠ There was a general agreement that we need to make better use of

computational tools to support the MoA description process. Literature mining needs computation otherwise we won't get the global picture (the workshop in Tubingen dealt with this topic, see following section 4.10.5.4).

➠ Gene expression analysis might be a useful tool to identify the biology associated with a specific physiological/toxicological condition, and this information might be used to fill the gap between MIE and AO, but it definitely needs IT support.

➠ We need a model for capturing the complexity of a pathway in terms of dynamic, kinetic and quantitative relationships between the different events and actors; verbal description alone is not sufficient for this purpose.

➠ We also need computer simulations to identify the most probable scenarios. Skin sensitisation could serve as a model with sufficient data and kinetics available.

➠ We could make more use of statistics; though statistical relevance does not reflect physiological relevance it might give probabilities for prioritisation.

Day 2

The second day focused on specific AOP-related **SEURAT-1** research project activities and needs. A representative from each project was asked for a short presentation on the project's MoA/AOP-related activities and how they fit into the overall **SEURAT-1** framework.

Maurice Whelan opened the session, referring again to the proof of concept objective. He listed various purposes for which systems might be designed and then highlighted the need for designing rational integrated systems to capture a specific MoA as a starting point for performing quantitative prediction for risk assessment purposes.

Questions for the second day were about (i) available and desirable MoAs/pathways and necessary features for further pathway elaboration, (ii) the practical application of theoretical MoA knowledge within **SEURAT-1** projects, (iii) which project activities can actually contribute to AOP development, and finally (iv) what concrete things should be done next.

Ian Cotgreave, co-chair of the **SEURAT-1** Scientific Expert Panel, confirmed the need to move on to the second step of the proof of concept as we are almost halfway through the work programme of the **SEURAT-1** Research Initiative.

'ToxBank: Data-Mining and Ontologies for the Identification of AOPs' (Christoph Helma): Key message: ToxBank provides initial support for the identification of AOPs and will develop additional tools in collaboration with the MoA working group.

Christoph Helma presented on how ToxBank can support MoA development and data storage. The subsequent discussion stressed the importance of having a solid knowledge base for practical work. Toxbank also can facilitate collaboration between projects and knowledge dissemination. A wiki-like tool could help to refine and evaluate elaborated MoA/AOP's through a crowd-sourcing process.

'MOA-Based Selection of Reference Compounds' (Jeff Wiseman): Outstanding issues in understanding MOAs were highlighted:

- Are adipocytes a dominant cell type for steatosis? In cases of steatosis induced by ER or LXR/PXR agonists this is probably the case.
- Especially in the case of chemically reactive toxicants it is not clear whether the frequently cited ROS mechanism is quantitatively important.
- Why do some toxicants cause fibrosis and others not? What is the signalling process that makes this distinction?

Jeff Wiseman added some words on the MoA-based selection of reference compounds. He mentioned the high promiscuity of many compounds; that is, one compound is likely to cause various adverse effects. The criteria for the selection of a positive control need to be identified; e.g. may a positive control cause only one specific adverse outcome, or is some degree of 'chemical promiscuity' allowed?

'The COSMOS MoA/AOP Strategy' (Steve Enoch): Steve Enoch presented on how knowledge of the chemistry related to the MIE can be used to develop *in silico* profilers for category formation. The identification of mechanism-based profilers requires an extensive and time-consuming literature search.

The resulting discussion focused once again on the need for better communication and integration between **SEURAT-1** partners and the use of computational tools for data mining.

Chris Corton referred to a database hosted by EPA that reports the NOAEL value of repeated dose toxicity tests for many chemicals and could be of interest for COSMOS.

'Mechanistic Basis for Compound Selection in the *HeMiBio* and DETECTIVE Projects' (Mathieu Vinken): Key message: The pragmatic approach for contributing to the **SEURAT-1** AOP strategy starts by verifying the types of toxicity that could be potentially induced by cosmetic ingredients. To do so, the safety evaluations of about 220 cosmetic ingredients, issued by the Scientific Committee on Consumer Safety between 2000 and 2009, were systematically screened. Although generally safe, about 4% of the cosmetic ingredients concerned could potentially negatively affect the liver. Specifically, a small minority of cosmetic ingredients may induce steatosis or cholestasis. It should be stressed that this assumption is merely based upon a set of clinically relevant histopathological parameters and biochemical

read-outs. However, the outcome of this large-scale screening exercise suggests that efforts with respect to the AOP strategy within the **SEURAT-1** Research Initiative could be focused on these two specific types of liver toxicity.

The presented compound selection criteria were rated as being too outcome driven; further downstream events could be due to various MIE and are therefore less suitable for categorisation. It was debated again whether the model (a bioreactor) needs to mimic the AO *in vivo* to prove toxicity and how the bioreactor could be used to deduce the AO from an IE without really producing this AO.

'NOTOX and MoA/AOP-Case Acetaminophen' (Elmar Heinzle): Elmar Heinzle first mentioned 3D Spheroid production and then spoke about the NOTOX Joint Experiment with 2D HepaRG and acetaminophen.

'Repeated Doses Toxicity of Doxorubicin on human IPSCs Derived Cardiomyocytes: an Integrative Functional, Transcriptomics and Metabonomic Approach (Agapios Sachinidis): Posed questions were about timing and doses of exposure and correlation with *in vivo* data.

Participants of the workshop were reminded that DETECTIVE is looking for biomarkers, while *SCR&Tox* is characterising the model and AOP elaboration is done by other groups.

'Designing Integrated Systems for Repeated Dose Toxicity – Getting Started' (Alfonso Lostia): The proof of concept objective at the system level aims to develop an integrated testing strategy to associate a chemical with an MoA category in order to predict repeated dose toxicity. Following the MoA approach, as a first step would be a strategy to (i) design an *in vitro* test system to capture selected modes-of-action; and (ii) design treatment protocols to define *in vitro* exposure conditions related to repeat-dose toxicity. The key step is the selection and description of an MoA since a test system should be built in order to capture a specific MoA. Then, the most suitable cell model is selected together with a positive control, i.e. a chemical known to cause an adverse outcome through the specific MoA. Regarding the selection of concentrations to be incubated in the *in vitro* model and the exposure conditions, it is mandatory to also consider kinetic parameters, particularly the *in vitro* biotransformation and metabolic clearance, in order to test a limited number of *in vitro* treatment protocols and to help with the harmonisation and comparison of testing strategies developed by different laboratories.

'**SEURAT-1** ongoing Cluster Activities (Elisabet Berggren): Identifying project key deliverables contributing to the cluster-level objectives and establishing cross cluster collaborations is essential to achieving the added value from the **SEURAT-1** Research Initiative compared to the outcomes of the individual projects. The real success of **SEURAT-1** will be shown by achieving the proof-of-concept that safety assessment of a chemical can be based on an integrated system of alternative methods predicting its MoA.

Discussion

The final discussion did not exactly follow the posed questions as the day before.

➠ Initially the discussion centred on kinetic aspects and it was agreed that kinetics need to be considered in the design of a test system even if there are substantial practical difficulties for implementation.

➠ Consecutively experimental design was debated and noted that we must not aim at mimicking whole humans in an *in vitro* model.

➠ It was also discussed that we might not need to model repeated-dose toxicity *in vitro*. Once the underlying mechanism is understood, repeated exposure might not be necessary any more, because it could be predicted from single exposure.

➠ The importance of defining the purpose before designing a test system was stressed again.

➠ There are two principally different scenarios for MoA knowledge discovery and MoA knowledge application. The research approach and experimental design suitable for development and elucidation of MoAs is quite different from the approach that is suitable for the application of this knowledge to the design of an integrated test system.

➠ The participants agreed that further efforts must concentrate on the development of integrated test systems based on the MoA framework.

➠ Finally it was discussed which other MoAs, besides liver fibrosis and steatosis, would be useful to develop for the **SEURAT-1** cluster. First it will be necessary to identify those MoAs that are relevant for **SEURAT-1** research objectives and collect available knowledge on these pathways. An inventory of data should be built and hosted by ToxBank. Those MoAs with highest concern from a safety assessment aspect should be selected and further elaborated. These tasks will be assigned to the respective working groups.

Summary of the key points

➠ The MoA development has to be distinguished from the MoA application; these are two different processes and are likely to require different experimental designs.

➠ Developing an MoA is an iterative and interactive process, starting with an unbiased description of the knowledge available, followed by a targeted

refinement depending on the intended use (e.g. related to the specific purpose of the prediction system).

➠ Rational design of an integrated prediction system is dependent on having a sufficiently described MoA.

➠ The MoA description has to be specific and quantitative (defining elements such as threshold values, dose-response and response-response relationships, etc.) for full risk assessment.

➠ There is a need for:

- Harmonisation and standardisation of MoA descriptions;
- A common language (e.g. terms, definitions);
- Descriptive terms that help to characterise the dynamic relationship between actors and events.

➠ Computational models and tools are indispensable for supporting the MoA development process (e.g. for literature mining, quantitative assessment, simulation of probable scenarios, etc.).

➠ Kinetic considerations are important and need to be considered in the design of a test system even if there are substantial practical difficulties for implementation.

➠ Gene expression analysis is an underutilised tool for MoA development. It can help to identify underlying mechanisms and fill in knowledge gaps.

➠ It was agreed that embracing an MoA framework on which the **SEURAT-1** strategy is based is a valid way to proceed, and further efforts must concentrate on the best way to implement it.

Recommendations

➠ ToxBank to serve as knowledge base and provide the dissemination of MoA knowledge within the **SEURAT-1** Research Initiative.

➠ The creation of a wiki-type platform for the storage of MoA knowledge.

➠ The evaluation and potentially further refining of an elaborated MoA/AOP should be done by a crowd-sourcing process, where experts within the **SEURAT-1** Research Initiative act as reviewers.

Next Steps

➠ Development of a generic process for the design of predictive toxicity systems using MoA/AOP knowledge. This process is different from MoA/AOP development, which is based on literature research and experimental data of response-response (dynamic) relationships.

➠ Identification of MoAs that are relevant for **SEURAT-1** research objectives (mapping the **SEURAT-1** toxicity landscape/domain).

➠ Collection of the available MoA/AOPs that have been developed by third parties and making them available to the consortium.

➠ Adopt/build an ontology of toxicological terms to facilitate harmonisation in MoA development/descriptions.

➠ Building an MoA knowledge inventory/base, e.g. test-wiki that will be hosted by ToxBank to disseminate MoA descriptions.

➠ Identification of MoAs of concern from the safety assessment aspect (including evaluation of relevance of neurotoxicity endpoints). Prioritisation of AOPs that could substitute regulatory animal testing.

➠ Further elaboration of priority MoAs that have been identified by the Safety Assessment Working Group.

Participants

Chris Corton *(US EPA-NHEERL)*, Ian Cotgreave *(Astrazeneca, SEP)*, George Daston *(Procter & Gamble)*, Steven Enoch *(Liverpool John Moores University, COSMOS)*, Elena Fioravanzo *(S-IN Soluzioni Informatiche, COSMOS)*, Tilman Gocht *(University of Tuebingen, COACH)*, Elmar Heinzle *(Saarland University, NOTOX)*, Christoph Helma *(In Silico Toxicology GmbH, ToxBank)*, Magnus Ingelman-Sundberg *(Karolinkska Institutet, SCR&Tox, NOTOX)*, Branislava Rakic *(Cambridge Cell Networks Ltd, NOTOX)*, Agapios Sachinidis *(University of Cologne, DETECTIVE)*, Terry Schultz *(OECD)*, Michael Schwarz *(University of Tuebingen, COACH)*, Imran Shah *(US EPA)*, Mathieu Vinken *(Vrije Universiteit Brussel, HeMiBio, DETECTIVE)*, Kendall Wallace *(University of Minnesota)*, Catherine Willett *(The Human Society of the United States)*, Jeff Wiseman *(Pharmatrope, ToxBank)*, Elisabet Berggren *(JRC, COACH)*, Susanne Bremer *(JRC, SCR&Tox, DETECTIVE)*, Marina Goumenou *(JRC, COACH)*, Brigitte Landesmann *(JRC, COACH)*, Alfonso Lostia *(JRC, COACH)*, Jochem Louisse *(JRC, DETECTIVE)*, Milena Mennecozzi *(JRC)*, Alicia Paini *(JRC, COSMOS)*, Francesca Pistollato *(JRC, SCR&Tox)*, Anna Price *(JRC)*, Maurice Whelan *(JRC, COACH)*, Clemens Wittwehr *(JRC, COACH*, Andrew Worth *(JRC, COSMOS)*.

4.10.5.4 Exploring Existing Databases for Modes-of-Action of Repeated Dose Systemic Toxicity

Introduction

This **SEURAT-1** workshop, organised by the Mode-of-Action Working Group and COACH, was held from 13–14 November 2012 in Tübingen, Germany. The goal of this workshop was to develop a concept for mining the extensive information hidden in RNA expression databases from acute and subchronic toxicity studies for defining pathways relevant for toxicity. As outlined earlier (*Whelan & Schwarz, 2011*), the guiding principle of the **SEURAT-1** research strategy is to adopt a toxicological mode-of-action framework to describe how any substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure needed for safety assessment. Therefore, this strategy fundamentally relies on harvesting, generating and consolidating knowledge about mechanisms of repeated dose systemic toxicity.

The hypothesis behind the workshop was that much of the relevant knowledge for the selection of appropriate prototype modes-of-action to be investigated within **SEURAT-1** can be gained from publicly available databases. Databases such as the Japanese TG-Gates or DrugMatrix® developed within the National Toxicology Program (NTP) are believed to be of incredible value for such an approach, as these databases not only contain information about organ toxicities of a large number of test chemicals as a function of exposure time and dose, but also information about global gene expression changes *in vivo* and in human and rat hepatocytes incubated *in vitro* with the test chemicals. Based on this hypothesis, the aims of the workshop were: (i) to develop a strategy to define regulatory networks disturbed by chemicals upon repeated dose exposure; (ii) to derive hypotheses that can be tested with methods (*in vitro* tests) to be developed in **SEURAT-1**; and (iii) to discuss alternative approaches of data-mining that could complement existing approaches.

For this, a number of international experts from outside of the **SEURAT-1** Research Initiative were also invited, highlighting the 'think-tank'-character of the meeting. The idea behind this invitation was to discuss the current state of toxicity data-mining approaches and how they can be best used to support the development of highly innovative tools and methodology to ultimately support regulatory safety assessment, which is one of the **SEURAT-1** cluster level objectives. The structure of the following report does not strictly reflect the sequence of the workshop agenda; rather, the contributions are grouped according to three main themes that were covered during the workshop: (i) the use of '-omics' data for the identification of toxicity pathways; (ii) publicly available data bases and bioinformatic tools for data-mining; and (iii) the integration of data-mining approaches into **SEURAT-1**.

The use of '-omics' data for the identification of toxicity pathways

Michael Schwarz opened the workshop and prepared the foundation of the workshop by formulating the following basic hypotheses as a guidance for discussion:

- Any toxic insult will produce a change in the mRNA expression pattern in target cells (and often indirectly in surrogate organs, such as the liver).
- The development of an adverse outcome pathway (AOP) requires *in vivo* data, including '-omics' information. Linking molecular initiating events with apical endpoints causally is impossible based on *in vitro* data alone (due to limitations in the biological models). Consequently, the development of suitable (targeted) *in vitro* test systems is a second step and should follow the AOP development, which should be based on existing *in vivo* data.
- Mining of existing databases is a suitable tool for AOP development.

André Kleensang highlighted in his talk entitled '*Mapping of toxicity pathways based on '-omics' data'* that a shift from animal testing to a mode-of-action-driven *in vitro* testing strategy will finally lead to probabilistic risk assessment procedures. Transcriptomics and metabolomics are promising tools for extracting information about toxicity pathways, but there is still a need to reach consensus on how to map, annotate and validate a toxicity pathway. This would be part of the field of systems toxicology as part of the so-called human toxome concept (*Hartung & McBride, 2011*). The knowledge of toxicity pathways should guide the establishment of integrated testing strategies, which will then provide the data basis for future (probabilistic) risk assessment procedures. However, the crux is in combining different '-omics' data and their interpretation in terms of toxicity pathways, which is still very challenging (*Hartung et al., 2012*).

Heidrun Ellinger-Ziegelbauer presented case studies about liver toxicity and '-omics' and kidney toxicity and transcriptomics in her talk entitled '*Molecular pathways of organ toxicity – the added value of '-omics' techniques'*. The studies were based on *in vivo* experiments that were conducted under the umbrella of the EU FP6 project 'PredTox' (*Ellinger-Ziegelbauer et al., 2011*). The reference compounds in these studies were selected according to similar histopathological effects, such as nephrotoxicity, liver hypertrophy, and bile duct damage/necrosis. These endpoints were studied using a combined approach comparing '-omics' with classic toxicological data. This approach reveals that transcriptomics data delivered mechanistic hypotheses to explain classical observations, whereas metabolomics contributed confirmatory information and suggested a potential biomarker candidate for bile duct versus hepatocyte damage. In summary, mechanistic investigations delivered details to and confirmed '-omics' findings, and suggested three diagnostic tissue damage markers (increased conjugated bile acids in serum and urine as potential biomarkers for bile duct damage, and increased unconjugated bile acids in urine as putative indicators of intrahepatic cholestasis). Overall,

'-omics' analysis, especially transcriptomics, allows more detailed mechanistic interpretations of organ toxicity compared to classical toxicity parameters. Furthermore, compounds inducing liver and/or kidney damage in rats appear to affect gene groups representing similar pathways/functions (representing mostly acute stress responses). The specific genes representing these functions may or may not be the same (dependent on organ, toxicant, exact damage induced, etc.). Additionally, the outcome of experiments focussing on *in vitro* (primary cells) versus *in vivo* responses revealed that some pathways could not be studied *in vitro* due to the lack of respective cellular functionality (related to cell cycle progression representing hyperplasia, regeneration, inflammation and dedifferentiation).

In conclusion, the molecular pathways affected upon toxicant treatment, as revealed with '-omics' analysis, may represent (i) primarily adaptive responses, e.g. oxidative stress/DNA damage responses, (ii) adverse responses, or (iii) first adaptive then adverse responses, depending on the strength of their downstream effects, e.g. regeneration may not only replace damaged cells with new ones, but also induce more than the required cell proliferation (the distinction requires dose- and time-dependent analyses).

Michael P. Ryan presented another case study on kidney toxicity from the EU FP6 project carcinoGENOMICS ('*Insights from the EU CarcinoGenomics project: predictions, pathways, inter-laboratory genomics, in vitro-in vivo*'). Two proximal tubular epithelial cell models (the immortalised non-tumour human renal epithelial cell line RPTEC/TERT1 and the rat renal proximal tubular cell line NRK/52E) were selected and optimised based on morphology and characteristics, barrier function, genetic stability, transcription profile and metabolic profile. The cells were treated for up to 72 hours with reference chemicals, which were grouped into genotoxic, non-genotoxic and non-carcinogenic compounds, and responsive gene sets were identified for the different compound groups in both human and rat cells. A pathway analysis suggests that gene changes in human cells may be more relevant to known carcinogenic mechanisms. The data were compared with clinical data and, based on that, a loss of cilia in RPTEC/TERT1 cells was identified as a possible additional *in vitro* readout for carcinogens. Comparisons to known gene changes in human renal cancer resulted in a high number of commonly regulated genes. Gene expression data of the two *in vitro* systems matched remarkably well with rat *in vivo* data after exposure to ochratoxin (i.e., no evidence for species-specific effects of ochratoxin), suggesting an epigenetic model for this compound, potentially arising from ochratoxin binding to actin-filament. Overall, the results suggest that the renal *in vitro* models can be used for grouping chemicals into the above-mentioned categories.

George Daston presented in his talk entitled '*Using chemical structure-based toxicity databases for insight into molecular initiating events'* a strategy to group chemicals according to their modes-of-action, based on a combination of chemoinformatic tools and '-omics' information. He presented cases in which gene expression analyses were used to group chemicals according to their modes-of-action, underlining that these data are indicative for specific modes-of action. In addition, databases organised by chemistry can be further developed by

means of structure-activity-based relationships (*Blackburn et al., 2011*). This was exemplified in the establishment of an expert decision tree system covering the field of reproductive and developmental toxicology using publicly available databases and computational tools. This results in 25 major categories and multiple sub-categories, in which the highest level of confidence is represented by (i) similar chemical structures, (ii) identified (biological) molecular targets, and (iii) similar downstream effects. In conclusion, both approaches, i.e. chemoinformatic tools and bioinformatic interpretation of '-omics' data, are complementary: Chemical ontology can aid in assigning chemicals to groups with the same putative modes-of-action, and gene expression analysis will aid in grouping chemicals by mode-of-action. In addition, it is possible to estimate the size of the mode-of-action universe.

Publicly Available Databases and Bioinformatic Tools for Data Mining

This session focused on two publicly available databases: The DrugMatrix® database published by the US National Institute of Environmental Health Sciences (NIEHS) in its National Toxicology Program, and the TG-Gates database published by the Japanese National Institute of Biomedical Innovation. Practical examples were shown highlighting the vast amount of knowledge that is stored in these databases. In addition, the eTOX project was presented, in which, besides others, both of the aforementioned databases are being used for the development of expert systems allowing the *in silico* prediction of toxicities. An overview of publicly available resources for data mining, which were used in the various presentations of this workshop, is given at the end of this section.

'*Resources I: The DrugMatrix® database'* was the title of the talk given by Scott Auerbach, in which he presented an overview of the DrugMatrix® database and showed some practical application examples of using the database and its integrated tools for short-term toxicity assessments. DrugMatrix® is a large-scale rat toxicogenomics database and analysis tool, which was originally owned by Iconix Pharmaceuticals and Entelos, Inc. and was acquired by the National Toxicology Program (NTP) in 2010. No data for these resources were generated by NTP. The motivation of acquisition was to make the computational and data resources freely available to the public without fee, to facilitate the integration of toxicogenomics into hazard characterisation, and to build a bridge between traditional toxicology and the US Tox21 research program. The database consists of several structural components, combining literature data of drugs about toxicity and biological pathways with data covering various biological profiles such as gene expression, pathology and pharmacology of hundreds of chemicals. It contains data of approximately 700 short-term toxicity studies (0.25 to 5 days) in male SD rats, in which 637 compounds were studied at multiple doses, time points and tissues. The standardised DrugMatrix *in vivo* experimental protocol considers two doses (the maximum tolerated dose and the fully effective dose) and four time points (0.25, 1, 3 and 5, 7, or 14 days) with 3 rats per time point. The data are organised in different domains, each of which can be used as a source to search for mechanistic information; for example, the gene

domain (search individual genes to find out how they behave with different treatments), the chemical domain (search individual chemicals to identify structurally similar chemicals, gene expression perturbations, assay hits, etc.), the assay domain (search and display *in vitro* assay results and clinical chemistry/hematology results), the expression domain (view results for single expression studies), the pathway domain (explore over 130 different biological pathways), the histopathology domain (find details on specific histopathologies annotated in DrugMatrix), and others. The DrugMatrix data are available through an ftp-server (ftp://anonftp.niehs.nih.gov/drugmatrix) and are categorised into unprocessed microarray data, microarray data normalised by organ, individual animal toxicology data, *in vitro* screening data and chemical annotations. The functionality and analysis tools can be used to compare one's own datasets, for example to contextualise them relative to over 4000 expression profiles elicited by >600 well-characterised, phenotypically anchored prototype agents, or to visualise expression profiles on pathways. Advanced search tools allow filtering for drug/target interactions and to mine data relevant to one's own research.

A toxicogenomic assessment of a mixture of three brominated diphenyl ethers (used as flame retardants in objects including furniture) was presented as an example application (*Dunnick et al., 2012*). The goal was to identify the potential toxicological effects of this mixture by means of toxicogenomics. Therefore, a gene expression study was designed and the DrugMatrix database was used to extract knowledge about respective pathways of toxicity. Based on that, three toxicological effects were identified, namely steatosis, repro-related endocrine perturbations, and alterations in lipid homeostasis. Overall, the expression profile suggested that the brominated flame retardants may exacerbate metabolic syndrome, following an AhR or CAR/PXR related mode-of-action. Thus, the DrugMatrix database assisted in the formulation of a hypothesis that can be further tested through a targeted experimental approach.

Michael Schwarz gave an introduction into the Open TG-GATEs database (title of his presentation: '*Resources II: The TG-Gates database*'). The TG-GATEs database comprises results of 170 chemicals tested in rats, which were exposed to 4 doses (including control) and analysed over time (8 time points: 3, 6, 9 and 24 hours and 4, 8, 15 and 29 days). mRNA expression data are available for liver and kidney as CEL files (Affymetrix analysis), as well as data regarding histopathology. All chemicals were also tested in rat and human hepatocytes (4 concentrations; 3 time points). The data can be used for elucidating network structures, i.e. changes in gene regulation over time subsequent to exposure with a toxicant, which leads to an improved understanding of the toxicological molecular mechanisms. This was exemplified by a case study with bromobenzene (*Kiyosawa et al., 2010*). In a review, the TG-GATEs database was recently compared with the DrugMatrix database, and commonalities and differences between them were discussed (*Chen et al., 2012*). In conclusion, the TG-GATEs database can be mined to address the following tasks: (i) Identification of 'toxicity pathways' for individual compounds or for groups of compounds with the same or similar mode-of-action; (ii) comparison of responses in different organs (liver and kidney); (iii) comparison

of responses *in vivo* and *in vitro*; (iv) comparison of responses in different species (rat and human). Additionally, the information about target genes can be used to search for chemicals where underlying transcription factor networks were similarly affected (e.g. AhR/CAR/PXR battery).

Another application case of this database was presented by Johannes Eichner in his talk entitled '*Bioinformatic analysis of transcriptome changes produced by alkylating agents in rat liver: a pilot study based on data from the TG-GATEs database'*. Time-course expression data of 13 alkylating compounds were obtained from TG-GATEs, pre-processed and further analysed for the identification of compounds with similar temporal profiles of biological activity (i.e., grouping of chemicals based on their biological activities). Differentially expressed genes for the different compounds were detected in the first step. Subsequently, clusters of co-regulated genes over time were obtained from the mRNA profiles (using the bioinformatic tool EDISA; *Supper et al., 2007*) and corresponding regulatory sequences were mined for active transcription factor binding sites (with ModuleMaster; *Wrzodek et al., 2010*). A multi-objective evolutionary algorithm was used to search in each cluster for an optimal combination of transcription factors that form a *cis*-regulatory module. On this basis, transcriptional regulatory networks were reconstructed by integrating knowledge from the mRNA profiles and regulatory transcription factors. Biological networks, such as the inferred transcriptional network or pathways, were then visualised and overlaid with the transcriptomic data in order to inspect cell-specific network changes caused by chemical interaction (using InCroMAP; *Wrzodek et al., 2013*), which are indicative for certain cellular functions (or dysfunctions). Several pathways overrepresented among co-expressed genes were identified with this approach, and the inference of regulatory networks shows regulatory relationships between gene clusters. In the next step, these regulatory relationships will be further interpreted with respect to their biochemical meaning.

Olivier Taboureau provided an overview of the eTOX project, which is currently funded by the Innovative Medicine Initiative (IMI) Joint Undertaking (title: '*Data integration for in silico toxicity prediction: The eTOX approach*'). Overall, the project aims to integrate bioinformatic and chemoinformatic approaches for the development of expert systems allowing the *in silico* prediction of toxicities. For this, bioactivity data from publicly available databases (such as Drug Matrix, TG Gates, PubChem, etc.) were collected in a data repository. Data on toxicogenomics are being mined to (i) search for common inter-species variation responses to a compound using *in vitro* data, (ii) identify common variation responses to a compound between *in vitro* and *in vivo* data, (iii) group chemicals according to similar expression profiles, and (iv) relate gene expression with toxicological endpoint by means of a Gene Set Enrichment Analysis approach (GSEA). Furthermore, a protein-protein interactions (PPIs) network was developed to evaluate how protein complexes are involved in similar biological and toxicological endpoints, assuming that proteins causing the same or similar diseases tend to lie close to each other in function. First results show that a relation between time, dose and gene expression variation

for a majority of compounds can be seen in *in vitro* data. Furthermore, comparison of the gene expression data of *in vitro* hepatocytes from rat and human does not show a similar profile in most of the cases.

Summary Overview About Tools

Table 4.18 summarises the different databases that were presented and discussed during the workshop. Additional information can be found on the homepage of the eTOX-project (http://cadd.imim.es/etox-library/), which provides a comprehensive overview of and links to bioinformatic tools, databases and research articles in its library.

***Table 4.18** Overview of databases and tools that were discussed at the workshop.*

Databases	Short description	Link
ACToR	Online warehouse of the US Environmental Protection Agency of all publicly available chemical toxicity data	http://actor.epa.gov/
DrugMatrix®	Molecular toxicology reference database and informatics system from the US Department of Health and Human Services, National Toxicology Program	https://ntp.niehs.nih.gov/drugmatrix/index.html
DSSTox	Structurally searchable database of the US Environmental Protection Agency	http://www.epa.gov/ncct/dsstox/
HExpoChem	Biological targets for chemicals, exploration of protein networks (TU of Denmark)	http://www.cbs.dtu.dk/services/HExpoChem-1.0/
Micromedex	Micromedex® 1.0 from Thomson Reuters, clinical data on drugs	http://www.micromedex.com/products/hcs/
Nephromine	Public repository for renal gene expression studies (Compendia Bioscience, Inc.)	http://www.nephromine.org/
Open TG-GATEs	Publicly available database from the Japanese Toxicogenomic Project, National Institute of Biomedical Innovation, Japan	http://toxico.nibio.go.jp/open-tggates/search.html
PubChem	Validated chemical depiction information (PubChem Compound) and bioactivity (PubChem Bioassay) from the US National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/pccompound
ToxRefDB	Toxicity Reference Database, captures *in vivo* animal toxicity studies on various chemicals	http://www.epa.gov/ncct/toxrefdb/

Integration of Data-Mining Approaches into **SEURAT-1**

Experimental work conducted within the **SEURAT-1** Research Initiative is limited to *in vitro* approaches. Due to restrictions outlined in the call for proposals, none of the research projects will conduct *in vivo* experiments. However, the final outcome of the **SEURAT-1** Research Initiative should be testing methods and strategies that are fit for purpose to support regulatory risk assessment. Therefore, an important question is how the *in vitro* testing systems and *in silico* calculation methods developed in **SEURAT-1** can find regulatory acceptance, given the fact that they cannot be proven by means of parallel *in vivo* experiments. Under this perspective, the existing *in vivo* data become extremely important, as they provide the potential source for benchmarking the **SEURAT-1** testing methods, which are currently under development.

The third session of this workshop was therefore motivated by the question of how the approaches presented in the previous sessions could be integrated into **SEURAT-1**. Two potential points of contact were identified before the workshop took place: the cross-cluster mode-of-action working group, and the work on Adverse Outcome Pathways (AOPs) conducted within COSMOS. Representatives from both groups gave a short overview of current activities in their respective fields. In addition, taking into consideration that *in vivo* '-omics' data may be different when compared with *in vitro* data for a number of reasons, the importance of pharmacokinetics was also addressed in this session.

Brigitte Landesmann gave a report from the workshop 'Describing mode-of-action in liver toxicity using adverse outcome pathways', which was held in Ispra in October 2012 (i.e., one month before this data-mining workshop, see section 4.10.5.3 above). Besides others, the most relevant point for the data-mining workshop was that gene expression analysis is an underutilised tool for mode-of-action development. The potential for this analysis is seen in supporting the identification of underlying perturbations of biological pathways and, thus, filling the knowledge gaps in existing AOP descriptions. In an ongoing effort, the **SEURAT-1** Mode-of-Action Working Group is identifying and collecting modes-of-action and AOP descriptions that are relevant to the **SEURAT-1** research objectives.

Mark Cronin reported on the current activities regarding the development and description of Adverse Outcome Pathways (AOPs) within the COSMOS project. According to the OECD proposal for developing AOPs (*OECD, 2012*), the minimum information for an AOP is (i) the identification of the chemical-biological interaction (i.e., the molecular initiating event), and (ii) the understanding of the apical outcome elicited by the molecular initiating event. The identification of intermediate events between these two points depends on the level of knowledge about the outcome and confidence in the AOP. These intermediate events are the basis for hypothesis development and testing and, thus, must be experimentally quantifiable. Toxicogenomics may be very helpful in the identification of such intermediate events. The role of COSMOS in AOP development is to organise the chemistry involved in the

processes. Currently, COSMOS develops structural alerts for key events allowing mechanistic interpretation (such as covalent protein or DNA binding); these alerts are used to develop profilers, which allow the formation of categories by linking chemical structures with toxicity data (literature data on NOAEL). Extracting mechanistic information from existing databases such as DrugMatrix or TG-GATEs could support the development of these profilers. By this, COSMOS would help to identify highly targeted *in vitro/in chemico* assays that could be developed and used to provide evidence to support the toxicity pathways of interest.

Russell Thomas made a distinction between selective chemicals (with a limited number of biological targets and subsequent toxicity pathways) and non-selective chemicals (affecting multiple biological pathways) and illustrated the consequences of grouping chemicals into these two categories for risk assessment approaches in his presentation entitled '*Considerations in Relating In Vitro Assays to In Vivo Responses in a Risk Assessment Context*' (see also chapter 2.3). A statistical evaluation of the approximately 600 high-throughput *in vitro* screening assays challenged with the 300 phase-I chemicals of the US EPA ToxCast programme across 60 *in vivo* endpoints revealed that the high-throughput *in vitro* assays have limited capability of predicting *in vivo* hazards (*Thomas et al., 2012a*). However, the results from the high-throughput *in vitro* screening assays can be used for separating the chemicals based on their selectivity by grouping them according to the number of assays they are activating. The strategy for the selective chemicals would then be to derive their mode(s)-of-action from the functional properties of the activated assays, and derive the point-of-departure for risk assessment by calculating the respective blood concentrations based on computational *in-vitro-to-in-vivo* extrapolation methods, as well as the equivalent exposure concentrations by means of reverse dosimetry calculations. The strategy for the non-selective chemicals would be to derive the point-of-departure for risk assessment from the most sensitive *in vitro* assay. Comparing the LOEL values from this approach with *in vivo*'s LOEL reveals that the *in vitro*-derived values are slightly lower in most of the cases, indicating that the most sensitive *in vitro* assay from the ToxCast programme provides a conservative estimate of the point-of-departure. The integration of human dosimetry and exposure with the ToxCast high-throughput *in vitro* screening assays shows that a total of approximately 10% of the chemicals tested by the EPA ToxCast programme have *in vitro* biological activity at oral equivalent doses that overlap the most highly exposed subpopulation (*Wetmore et al., 2012*). Thus, the incorporation of dosimetry and exposure is essential for priority setting regarding toxicity testing of chemicals.

It has been demonstrated that *in vivo* transcriptomic changes have the potential to be used (i) for the identification of toxicity pathways, and (ii) to estimate non-cancer and cancer points-of-departure for use in quantitative risk assessments, as the lowest transcriptional benchmark dose values correlate with those for the traditional non-cancer and cancer apical endpoints for specific pathways (*Thomas et al., 2012b*). Similar relationships were obtained for a limited number of *in vitro* studies.

Discussion and Conclusions

Biological Models and Adversity: The functional characterisation of the biological models plays a pivotal role in ensuring their suitability for toxicity testing. The functional change in the cell line as compared with the *in vivo* situation must be known in order to ensure the relevance of the cell line for the mechanism of interest. There is no doubt that artificial cell lines are corrupted and using them requires mechanistic information that proves the relevance of the biological model. Furthermore, it is well known that the functionality of primary cells is affected subsequent to their isolation from the tissue. In summary, a biological *in vitro* model that can be used for all purposes in the context of toxicity testing does not exist and it seems to be impossible to generate such a master model. Therefore, the biological model must be selected dependent on either (i) the toxicological endpoint or (ii) the mechanism of action that needs to be addressed — that is, the purpose must be defined first to be able to select the relevant biological model. Addressing a particular mechanism of action implies comprehensive knowledge about the respective toxicity pathway as a prerequisite. However, a still unresolved problem is how to characterise the cell lines appropriately to ensure their fitness for purpose. In this regard, '-omics' data may be helpful to distinguish relevant cell lines from those that are not sensitive and, thus, not suitable to be used for the pre-defined purpose.

Another important point for discussion is the question of the definition of adversity for regulatory purposes, culminating in the demand for defining the appropriate point of departure for risk assessment: How can we feed *in vitro*-based dose responses into the regulatory decision-making context? Would it be sufficient to define a safe dose based on the most sensitive *in vitro* biological response, or do we rely on organ-specific NOAEL values? A pragmatic answer from the regulatory side is that the benchmark for alternative testing methods would be the 90-day *in vivo* study, i.e. the alternative approach must deliver equivalent information to what can be taken from the *in vivo* 90-day study in order to find regulatory acceptance. However, the crux is then in the term 'equivalent'. It is important to note that, generally speaking, the distribution of biological receptors for chemicals, as well as the pharmacokinetic of a chemical, is organ specific, but the mode-of-action is not necessarily organ specific. Therefore, would it be enough to know when certain pathways are affected, or is it still necessary to look at apical endpoints? Would '-omics' data be acceptable (not only gene expression but also proteomics, etc.) or do we always need to have the related phenotypic information? Would the absence of toxicity be acceptable given that the biological model is relevant for a mechanism of interest (see above), and if so, how could it be proven? A general approach probably cannot be defined at this point, as procedures applicable to industrial chemicals are generally different from those applicable to pharmaceuticals. Pharmaceuticals are designed for a specific purpose with clearly defined cellular targets and, therefore, cannot be evaluated independent from the organ level. In the case of industrial chemicals it could be sufficient to elucidate the most sensitive biological responses and define the applicability domain based on chemoinformatics.

Data-Mining for Deriving Mechanistic Information: There was a broad consensus among the workshop participants that mining the publicly available databases is an important source of information for deepening our mechanistic understanding about molecular processes ultimately leading to toxicity. In the context of Adverse Outcome Pathways, information from the databases can be very useful for defining the intermediate events, as well as the molecular initiating event(s). The time-resolved gene expression data are of particular importance for elucidating the intermediate events and can potentially show if different upstream events would lead to the same adverse effects. The interpretation of the gene expression pattern is, however, equally important as its description, i.e. it is crucial to understand why some genes are relevant for particular pathways and others are not. Under this perspective, transcriptomics can be taken as a first step towards determining a toxicity pathway, which needs to be confirmed by corresponding changes at the protein level.

There is, however, a lack of specific expertise needed for database mining among the members of the **SEURAT-1** Research Initiative, and the tasks outlined above are not foreseen in any of the work programmes of the **SEURAT-1** projects. The invited external experts agreed that they could provide some support and, thus, database mining was identified as an important field for collaborations with related international research projects (such as the eTOX-project). As a prerequisite, such collaborations should meet the clearly defined aims of the **SEURAT-1** projects, e.g. the identification of intermediate key events for a particular Adverse Outcome Pathway for a well-defined class of chemicals. In this regard, COACH should play an active role for facilitating the interactions between the **SEURAT-1** projects and other international research activities.

Pharmacokinetics/Dosing: The field of pharmacokinetics is certainly a gap within **SEURAT-1**, even though the COSMOS project has some capabilities in biokinetic and PBPK modelling and can assist the experimentalists in designing their experiments and determining internal exposures (doses at target organ level) necessary for eliciting the effects. Furthermore, the parameters for pharmacokinetic modelling are well known for most (if not all) of the selected standard reference compounds. In fact, the compound selection strategy was to capture as much knowledge as possible for a few chemicals covering important modes-of-action. However, the question about the appropriate dosing regimes needs to be carefully addressed, taking into consideration (i) the desired read-out, (ii) the chemical properties (how does it distribute in the *in vitro* testing system?), and (iii) the biological activity (is the parent compound the agent or does it require metabolic activation?).

Summary Remarks from the Safety Assessment Working Group

The Safety Assessment Working Group had a workshop in parallel to the Data-Mining Workshop of the Mode-of-Action Working Group. A detailed report can be found elsewhere

(see section 4.10.8.2), but important points that were highlighted during a joint meeting at the end of both workshops are summarised in the following paragraphs.

Risk assessors need clearly defined protection goals and this would be a starting point for designing proof-of-concept case studies based on testing methods to be developed in the **SEURAT-1** Research Initiative. Chemicals with well-known modes-of-action and pharmacokinetics should be taken from the **SEURAT-1** list of reference compounds. In terms of AOPs, intermediate key events should be identified and testing systems need to be developed addressing these events. These testing systems should be challenged with the selected reference compounds as positive controls, as well as testing compounds (ideally cosmetic ingredients). The sensitivity of the testing systems is a very important point and needs to be investigated. Finally, acceptable daily doses of the testing compounds need to be derived.

Such a strategy could be one way to continue joint efforts of the **SEURAT-1** projects. A second strategy could be the use of '-omics' data for read across within a regulatory context, which was recently accepted by European regulatory bodies as an alternative testing strategy (*ECETOC, 2012*), and first examples for successful applications exist.

Recommendations

The definition and design of cross-cluster case studies appear to be the way forward for the **SEURAT-1** Research Initiative as a whole. In general, the approach for designing these case studies should include the following steps, taking into consideration the challenges with respect to the selection of appropriate biological *in vitro* models and the general agreement among the **SEURAT-1** Research Initiative to adapt the AOP approach for the formulation of case studies in a proof-of-concept effort:

1. Definition of the protection goal (Safety Assessment Working Group);

2. Selection of prototype AOPs relevant for the protection goals (Mode-of-Action Working Group);

3. Identification of appropriate biological models (Stem Cell Working Group);

4. Definition of standard reference compounds as positive controls;

5. Development of integrated testing strategies combining several methods developed within the **SEURAT-1** Research Initiative.

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4.10.5.5 Compilation of Modes-of-Action relevant for SEURAT-1

Description of the Task

The Mode-of-Action Working Group (MAWG) was assigned the task of identifying relevant MoAs and the collection of available knowledge (without going too much into detail) on these identified MoA/AOPs. The Safety Assessment Working Group will then identify the MoAs of concern from the safety assessment aspect for further elaboration by the MAWG.

Approach

A teleconference with the members of the MAWG was held on 7 January 2013 and it was decided to collect not only the expertise from within the working group but to ask all **SEURAT-1** partners for their opinion on relevant adverse outcomes for the organ of their expertise and the availability of mechanistic data for these adverse outcomes.

For each organ (besides muscle tissue) a lead and contact person was identified:

- Liver: Mathieu Vinken;
- Nervous System: Anna Price and Andrè Schrattenholz;
- Kidney: Paul Jennings;
- Heart: Agapios Sachinidis;
- Skin: Nick Pearson.

The results of the short questionnaire that was sent to all collaborators are summarised in the following overview. This summary was submitted to the Safety Assessment Working Group for the selection of the pathways with the highest concern from the safety assessment aspect.

Results

The following list was compiled for the liver:

- Fibrosis: Protein alkylation (elaborated pathway);
- Steatosis: LXR activation (elaborated pathway); other mechanisms: (i) decreased mitochondrial fatty acid beta-oxidation; (ii) microsomal triglyceride transfer protein (MTP) inhibition leading to decreased VLDL excretion; (iii) increased endogenous fatty acid synthesis; (iv) enhanced fatty acids uptake;
- Cholestasis: 'DETECTIVE' has decided to focus on this adverse outcome and the respective AOP has already been elaborated and will be published soon by Mathieu Vinken;
- HC carcinoma: Literature available;
- Hepatocellular injury: Adverse outcome Necrosis/apoptosis (literature available);
- Biliary hyperplasia + inflammation: Literature available.

The following list was compiled for the heart:

- Arhythmia: Conductance disturbance, ions channels pathways;

➠ Heart failure: Contractility impairment – mechanistic aspects and involved pathways;

➠ Effect on contractile protein or ions channels pathways: Literature available;

➠ Mitochondria associated metabolic pathways (e.g., Oxidative phosphorylation, ATP synthesis): Literature available;

➠ Stress-associated pathways (e.g., ROS-associated pathways): Literature available.

Reabsorption disturbance via stress response pathways was identified with respect to the kidney (literature available): Renal dysfunction is heavily dependent on which part of the nephron one is looking at. We work mostly on the proximal tubule region as it is the most susceptible to xenobiotic injury. This area specialises in reabsorption and thus anything that interferes with reabsorption would be considered a dysfunction. The stress-response pathways that can lead to cellular deregulation are not necessarily different to those in other cells, and include oxidative stress (Nrf2), ER stress (unfolded protein response), DNA damage response (p53), etc.

The following list was compiled for the nervous system:

➠ Mitochondrial damage, oxidative and nitrosative stress: Literature available.

Brief description: This MoA contributes to the etiology of many neurological disorders in the developing and aged/mature central nervous system, including acute trauma such as ischemia and hyperoxia, as well as chronic diseases such as Alzheimer's and Parkinson's diseases. The oxidative/nitrosative stress triggers the modification of specific proteins, including those involved in apoptosis, proteolysis and protein (mis)folding. Several disorders, including stroke and Parkinson's disease, are associated with inactivating modifications of antioxidant enzymes themselves, thus compromising antioxidant defences. Moreover, the regulators of antioxidant defences such as nuclear erythroid 2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) could be the key biomarkers of this pathological state.

➠ Excitotoxicity (CNS): Literature available.

Brief description: Neurons are damaged and killed by excessive stimulation by neurotransmitters such as glutamate. This occurs when receptors for the excitatory neurotransmitter glutamate, NMDA and AMPA, are overactivated and high levels of calcium ions enter the cell. A Ca^{2+} influx into cells activates a number of enzymes, including phospholipases, endonucleases and proteases

such as calpain. These enzymes damage cell structures such as components of the cytoskeleton, membrane, and DNA. Excitotoxicity is involved in stroke, traumatic brain injury, neurodegenerative diseases of the CNS such as multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, alcoholism or alcohol withdrawal, and Huntington's disease.

➠ Neuroinflammation: Literature available.

Brief description: Neuroinflammation is characterised by increased glial activation, pro-inflammatory cytokine concentration, blood-brain-barrier permeability and leukocyte invasion. One key player that is believed to drive this neuroinflammatory process is interleukin (IL)-1 beta, a pro-inflammatory cytokine that is up-regulated in Alzheimer's disease, Parkinson's disease, multiple sclerosis and other neurodegenerative disorders. IL-1 beta signals through the type I IL-1 receptor leading to NFkB-dependent transcription of pro-inflammatory cytokines (tumour necrosis factor (TNF)-alpha, IL-6 and interferons) and neutrophil-recruiting chemokines (CXCL1 and CXCL2) in glia. Additionally, a high amount of nitric oxidate is produced by microglia as a result of iNOs expression. All together it leads to neuronal cell death.

➠ Protein aggregation (mis)folding and inhibition of the ubiquitin-proteasome system (aberration in protein degradation): Literature available.

Brief description: Intracellular deposition of aggregated and ubiquitylated proteins is a cytopathological feature of most neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease and spinocerebellar ataxias. To avoid this abnormal deposition of proteins the cells recruit molecular chaperones to suppress aggregation and the ubiquitin/proteasome pathway (UPP) to remove the aggregate-prone proteins. The UPP eliminates mutated or abnormally modified proteins by degradation, in order to prevent the aggregation, which often form intracellular inclusion bodies.

➠ Organophosphate-induced delayed polyneuropathy (OPIDP): Literature available.

Brief description: The condition is associated with sensory-motor axonal degeneration of the peripheral nerves and spinal cord. The lesion, known as Wallerian-type degeneration, is followed by myelin degeneration of distal portions of the tracts of the central and peripheral nervous system. It is a rare toxicity resulting from exposure to certain organophosphorus esters. Neuropathy target esterase (NTE) is thought to be the target of OPIDP. The ratio of inhibitory powers for acetylcholinesterase and NTE represents the crucial guideline for the safety of using OPs.

➠ Voltage-gated sodium channels (VGSC) mediated neurotoxicity: Literature available.

Adverse outcome: Increased sensitivity to external stimuli, fine tremor, prostration.

Brief description: Short- or long-lasting VGSC modifications, altered neuronal firing rates and disrupted neuronal networks and pathways. Studied in multiple species *in vitro* and *in vivo* as a wide variety of compounds are known to interact with VGSCs (e.g., pyrethroids).

➠ Damage of the BBB: Literature available.

Brief description: Damage can be measured by a decrease of TEER and an increase of permeability, leading to the leakage of BBB, (e.g., induced by TCDD mediated by the aryl hydrocarbon receptor). The model and endpoints are well established. Increased toxicity in the CNS as the BBB is leaking.

➠ Activation of the aryl hydrocarbon receptor (AhR): Literature available.

Adverse outcome: Neurobehavioral abnormalities associated with both cognitive and locomotor systems.

Brief description: AhR is a key receptor involved in signalling the teratogenic and carcinogenic properties of a dioxin. There are lots of studies (*in vivo* and *in vitro*) showing the molecular mechanisms and the signalling pathways involved in TCDD-induced neurotoxicity. TCDD is an exogenous ligand of the AhR which binds to this receptor with a high affinity.

Skin sensitisation is an elaborated AOP for the skin. Furthermore, the methods survey in November 2012 identified one activity dedicated to muscle tissue, which is the investigation of mytotoxicty of statins with 'cell death' and 'effect on cell proliferation' as endpoints. The respective MoA is:

➠ Rhabdomyolysis: Literature available.

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4.10.5.6 Using Mechanistic Information in Developing the Concept of the Adverse Outcome Pathways Relevant to Human Neurotoxicity Evaluation

Introduction

One of the actions that have been agreed at the **SEURAT-1** MoA workshop in October 2012 in Ispra was the identification of MoAs/AOPs that might be relevant for **SEURAT-1** research objectives and the collection of available knowledge on these pathways (see section 4.10.5.3); this task has been assigned to the Mode-of-Action Working Group (MAWG). In pursuit of this action, an expert workshop on neurotoxicity was organised by the JRC in collaboration with the MAWG and COACH, which took place from 21–22 March 2013 in Ispra, Italy.

While there are a large number of cellular and molecular processes known to be critical to proper development and function of the central (CNS) and peripheral nervous system (PNS), there are relatively few examples of a comprehensive understanding of pathways leading from chemical exposure to an adverse outcome in the CNS or PNS. The lack of AOPs hampers the development of alternative testing methods for *in vitro* neurotoxicity evaluation and limits the predictive ability of high-throughput chemical testing.

In assessing neuropathology of CNS and PNS, additional complexity is added by the various windows of susceptibility during different life stages at which the organism is more vulnerable when exposed to the chemical such as developmental neurotoxicity (e.g. neurodevelopmental disorders, cognitive decline, IQ loss, autism etc.) or aging linked neurodegenerative disorders (Parkinson Disease, Alzheimer Diseases etc.). Moreover, attention has been driven to species-specificities as signalling pathways might differ between humans and rodents.

The overall aims of this workshop were:

- to identify pathways relevant to various human neuropathologies during adulthood and discriminate them from more vulnerable life stages such as development and ageing;
- to prioritise the identified pathways of neurotoxicity taking into consideration the relevance, the amount of available knowledge, but also the availability of reference chemicals and cell models and endpoints;
- to build working groups for the further development of selected AOPs.

The invited experts were provided beforehand with the background documents and a template (aligned to the OECD template) that they have used to present the chosen AOPs relevant for neurotoxicity evaluation.

Outcome

The following AOPs have been identified for further development by the participants according the OECD guidance:

1. NMDA-R over-activation

- MIE: binding to NMDA-R protein and its prolonged activation
- Key Event 1: influx of Na^{+} and Ca^{2+}
- Key Event 2: prolonged cell depolarization
- Key Event 3: massive influx of calcium
- Key Event 4: mitochondrial dysfunction
- Key Event 5: activation of calcium dependent enzymes; e.g calpain
- Cellular Response: damage of cytoskeleton, induction of apoptosis and/or necrosis
- Organ Response: Neurodegeneration
- Organism Response: Loss of sensory, motor and cognitive function

2. NMDA-R antagonism (relevant to both neurotoxicity and developmental neurotoxicity)

- MIE: binding to NMDA-R and its inactivation (e.g. by lead)
- Key Event 1: reduction of Ca^{2+} entry
- Key Event 2: phosphatase Activation
- Key Event 3: de-phosphorylation of proteins e.g. involved in synaptic plasticity
- Key Event 4: disruption of BDNF secretion and TrkB signaling
- Cellular Response: impairment of activity-dependent synapse formation: decreased number of synapses
- Organ Response: disturbed network formation
- Organism Response: learning and memory deficits; possibly schizophrenia

3. Neuroinflammation

- MIE: multiple
- Key Event 1: microglia and astrocyte activation
- Key Event 2: ROS and NRS production
- Key Event 3: production of pro-inflammatory cytokines
- Cellular Response: cytoskeleton instability/damage, synapses loss
- Organ Response: neurodegeneration
- Organism Response: contribution to neurodegenerative disorders (e.g. PD/AD, ALS and aging)

4. Energy depletion (mitochodria dysfunction)

- MIE: multiple (eg. NMDA-R prolonged activation, MTPT, Paraquat)
- Key Event 1: ROS production
- Key Event 2: oxidation of DNA, proteins and lipids
- Key Event 3: decrease mitochondrial function (e.g. membrane potential, ATP level)
- Key Event 4: PARP-1 activation with ATP/NADH depletion, triggering of apoptotic pathways
- Cellular Response: apoptosis
- Organ Response: neurodegeneration
- Organism Response: contribution to various neuropathologies

5. Energy depletion (mitochondria dysfunction) using NPC model of aging

- MIE: ROS-dependent formation of NAD+
- Key Event 1: ROS production
- Key Event 2: oxidation of DNA, proteins and lipids

- Cellular Response: mitochondrial dysfunction, cytoskeleton instability
- Organ Response: neurodegeneration
- Organism Response: contribution to various neuropathologies

6. Delayed Neuropathy

- MIE: NTE inhibition
- Key Event 1: disruption of Ca^{2+} homeostasis
- Key Event 2: mitochondrial dysfunction
- Key Event 3: calpain activation
- Cellular Response: cytoskeleton disruption
- Organ Response: Wallerian degeneration
- Organism Response: PNP, ataxia, paralysis

7. Blocking of GABA-R Channels

- MIE: binding to GABA-R Channels and blocking its activity
- Key Event 1: reduced influx of chloride
- Key Event 2: increase in membrane excitability
- Cellular Response: alterations in firing
- Organ Response: altered neuronal network function
- Organism Response: acute and prolonged symptoms associated with convulsant state

8. SH-group binding-induced neurotoxicity and developmental neurotoxicity

- MIE: binding to SH groups in cysteine, transport through BBB by LAT1
- Key Event 1: binding to GSH
- Key Event 3: increase in ROS
- Key Event 4: Ca^{2+} influx
- Key Event 5: glutamate overload in synaptic cleft

- Key Event 6: reduced uptake of glutamate by astroctytes
- Cellular Response: decreased level of reduced gluthatione, damage of cytoskeleton
- Organ Response: alterations in neuronal connectivity and plasticity, migration inhibition
- Organism Response: loss of sensory, motor and cognitive function

Summary of the Key Points from Discussion

- Kinetics, concentrations (low and high), time of the exposure (acute and chronic) and species-specificities need to considered in the design of a test system;
- Selected *in vitro* model should represent not only different neuronal cell types but also glial cells (astrocytes, microglia and oligodendrocytes) as they (especially astrocytes) play an important role in the response to chemically induced neurotoxicity;
- All existing neuroblastoma cell lines do not form functional synapses, so there are not suitable for functional assays, such as electrical activity measurements;
- According to the OECD guidance, 2 anchors should be identified for AOP definition:

Molecular Initiation Event (MIE) and Adverse Outcome (AO);

- AOP development can be based on the key events that lead to the well defined neuropathology, without identification of the specific MIE;
- Gaps in the knowledge should be identified;
- For regulatory purposes it should be proved that *in vitro* tests produce the same information as the *in vivo* studies.

The participants pointed out that an approach based on individual AOPs has strong limitations in being able to identify small number of positive 'hits' (neurotoxic compounds) and they proposed to identify 'Converging Key Events' (CKE) for Neurotoxicity that are common to many individual AOPs. Those CKEs could serve as the basis for practical assays development and lead to increased number of identified neurotoxicants even if their mechanisms of toxicity are mediated by various pathways of toxicity.

Next Steps

➠ Selection of the AOP by the JRC that will be further developed in detail according to the OECD template.

➠ Establishment of assay(s) in the JRC laboratory based on the key events of the selected AOP for neurotoxicity testing of the chemicals

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4.10.6 Biokinetics Working Group

Alexandre R.R. Péry

The Biokinetics Working Group was reinitiated during the 2013 **SEURAT-1** Annual Meeting. The meeting was attended by 30 people (15 more than for the initiation last year, with a large representation from outside COSMOS).

We started with a presentation of the achievements of toxicokinetic modelling highlighting a case study on acetaminophen. The general strategy we presented was the following: Firstly, *in vitro* tests to assess and quantify *in vitro* toxicity should be analysed with fate and effects mathematical models (referred to as toxicodynamic (TD) models). To achieve this, concentration measurements in the system in order to build a relevant, even simple, model of the dynamics of exposure in the system are required. The modelling of the *in vitro* systems can assist in both extrapolating the results to *in vivo* situations and addressing which parameters in the systems should be controlled or modified for more accurate results. Secondly, toxicokinetic models such as PBPKs would allow us to relate a scenario of exposure for humans (repeated dose at regular intervals, for instance) to an expected profile of concentration at a target level (for example, in the liver, in the case of acetaminophen). The calibration of such models can be performed through alternative methods (QSAR models, but also *in vitro* testing, especially to assess metabolism rates). The coupling of the PBTK and TD models facilitates the derivation of a dose response at organism level. For this coupling, the concentration and

effects are measured over time. In particular, effects considered at cell level are correlated to cell population dynamics, measured as the number and distribution of the cells in different phases.

Two of the expectations of the **SEURAT-1** projects are of relevance to biokinetics: (i) providing tools to analyse *in vitro* systems, including microfluidic ones; (ii) using PBPK modelling to relate *in vivo* doses of exposure to *in vitro* exposure concentrations. We discussed how both can be addressed. Regular work meetings will be organised on *in vitro* biokinetic modelling of the systems in development within the projects, to exchange information about modelling developments and to provide some training and support. At the same time, the availability of toxicokinetic parameters for the compounds under investigation in the **SEURAT-1** projects will be assessed. Moreover, a generic PBPK model (coded in R) based on the one developed for acetaminophen will be provided to the **SEURAT-1** partners. The meetings should take place twice a year, and the first one is planned in Paris in September 2013.

In conclusion, the Biokinetics Working Group will support the modelling of *in vitro* experiments and develop tools to relate *in vivo* predictions to *in vitro* data. We are on the way to the paradigm shift we identified last year from pure experimental approaches to guided model-based ones.

4.10.7 Stem Cell Working Group

Christian Pinset

SEURAT-1 has initiated a Stem Cell Working Group to support the development of good stem cell culture practice principles and promote best practice in the development of standardised cell-based assays for predictive toxicology purposes across the **SEURAT-1** Research Initiative projects.

The objectives of the working group are: (i) the identification of key areas of scientific development where review focusing on predictive toxicology would be helpful to the **SEURAT-1** objectives; and (ii) the definition of key criteria and steps required in the development of *in vitro* cell predictive toxicity assays adapted to high-content and high-throughput methods. In the development of stem cell-based toxicity assays, a range of cell lines are currently employed in different and rapidly developing protocols. Nevertheless, we are still exploring the use of human pluripotent stem cells as biological resources for predictive toxicology. The study and definition of protocols for differentiation are in their infancy. This complex matrix makes it very difficult to draw comparisons across work in different laboratories and thus standardisation is very challenging.

To tackle this reality, the Stem Cells Working Group has collected information from members of the **SEURAT-1** Research Initiative projects involved with cells, differentiation and amplification

protocols, freezing techniques and quality controls. As expected, the catalogue of different stem cells and progenies is diverse. Maintenance of human pluripotent stem cells is obtained in the presence of mouse feeder cells or in complete defined media. Some teams develop their own systems of differentiation and others get cells from providers. The traceability and the system of quality control are complex and far away from being homogeneous. Sometimes, differentiation protocols are not completely stabilised. In certain systems of differentiation, it is still difficult to isolate cell progenies that are committed in a specific lineage, while still maintaining a high proliferating capacity. Obtaining such cell progenies permits the construction of cell banks and facilitates definition of cell assays for HCS and HTS. Until now, most of the developed cell-based assays focus on acute toxicity.

It is now time to begin discussion of quality control applied to cells produced for predictive toxicology assays and to optimise and simplify these controls. As part of this, it will be important to introduce functional tests. The definition of 'clean' IPS cells is achievable, and the introduction of more automation in cell culture techniques and the improvement of freezing techniques are becoming more widely available.

To conclude, the **SEURAT-1** Stem Cell Working group is working on collecting data and to present questions that must be addressed. Our next objectives are to guide the exchanges of different protocols and comparisons of cellular systems and their responses to toxic agents, as well as repeated dose exposure protocols. The ultimate goal is to select the most predictive cell systems for toxicology.

4.10.8 Safety Assessment Working Group

Andrew White, Derek Knight

4.10.8.1 Introduction

The Safety Assessment Working Group was initiated to bridge the gap between the safety assessment decision making needs and the innovative predictive systems being developed within the cluster. The aim being to harness the mechanistic outputs of the **SEURAT-1** approach and to support the **SEURAT-1** cluster objectives with an emphasis on how the emerging science can best impact and reshape current risk assessment practice, in particular objective 3: To demonstrate proof-of-concept at multiple levels, from theory to application, focussing on the application level to identify pragmatic solutions in the use of information derived from predictive tools to support safety assessment processes and decisions.

The initial work of the Safety Assessment Working Group has focussed on the broader context devising a conceptual framework as a rational but flexible integrated assessment strategy to 'pull together' the various elements of the **SEURAT-1** work and provide context for where the various strands of work being undertaken can be aligned and incorporated. It also enables engagement from within the projects on where additional data could be further utilized. The

approach at its core focuses on modes-of-action/Adverse Outcome Pathways that, when fully developed in detail, will describe how to assess a substance for repeated-dose systemic toxicity (see chapter 3). Within **SEURAT-1** the aim is to set the framework and show through the application of specific case studies how mechanistic risk assessment could proceed, and in doing so, make a start in filling in the details of the strategy and identify knowledge gaps and areas for further development. The gold compounds identified provide an initial basis for work to proceed as they provide known human relevant toxicants that we can benchmark the framework against. Nevertheless we need to remain clear that we are not risk assessing for pharmaceuticals and therefore we need to be able to generalise the approach to additional compounds and more generic chemistries.

4.10.8.2 Workshop on 'The Development of Case Studies to Define Fit for Purpose Safety Risk Assessment of Repeated Dose Systemic Toxicity'

The objective of this first meeting of the Safety Assessment Working Group (13–14 November 2012 in Tübingen, Germany) was to explore how such an overall Integrated Assessment Strategy could be formulated. To focus the discussion, there was a primary aim to develop concepts for case studies to assess the ability of chemicals to cause repeated-dose systemic toxicity using data and prediction models from across the **SEURAT-1** Research Initiative. Two case study scenarios were considered, addressing different approaches to safety assessment:

- An *ab initio* assessment using the Integrated Assessment Strategy developed by **SEURAT-1**, as a 'stretching target' that would highlight gaps for future development and illustrate overall progress made in **SEURAT-1**.

- Make use of information from the **SEURAT-1** *in vitro* molecular screening and '-omics' data, as well as computational models to strengthen the weight of evidence approach and to improve the robustness of a read-across or chemical categorisation and validating the case. This is a realistic target within **SEURAT-1** and will immediately demonstrate the useful value of the work of the cluster for chemical safety assessment and at the same time reducing animal testing.

There were useful discussions at the Workshop inspired by the agenda:

- Setting the Scene: welcome and introduction to Safety Assessment Working Group and goals of the Workshop.

- Perspectives on developing *in vitro* points-of-departure for chemical risk assessment

➠ Outcomes from the workshop 'Describing mode-of-action in liver toxicity using adverse outcome pathways'.

➠ Adverse Outcome Pathway example.

➠ What are the use cases/scenarios for application of data from **SEURAT-1** covering differing needs across all stakeholders including regulatory and commercial decision makers?

➠ Initial discussion on developing a conceptual framework as a rational integrated assessment strategy incorporating modes-of-action/Adverse Outcome Pathways.

4.10.8.3 Indicative Rational Integrated Assessment Strategy for SEURAT-1

At the Workshop the 'infrastructure' of the 'conceptual framework' that could constitute the 'rational integrated assessment strategy' was explored, and outputs including the key elements described below were defined. The aim is to stimulate debate and start the process of defining which of these components are workable and how they can be used to both define and quantitate the dose response of both substances with specific modes-of-action and also of more general chemistries. There is an expectation that this will be refined as more information becomes available.

➠ The 'protection goal' of the conceptual framework should be clarified at the beginning when the assessor is deciding on the approach for a particular case; i.e. what the overall prediction is to be used for so it can be decided what is 'fit for purpose'. By articulating the protection goal this can focus subsequent data needs on what will be measured to obtain value-relevant outputs for risk assessment. It could be for use in a risk assessment by industry of an ingredient in a cosmetic product for example by ensuring that the observed response remains within a deemed homeostatic range, for priority setting or to provide a non-animal approach to assess systemic toxicity to meet a particular regulatory requirement dependant on the needs of the stakeholders.

➠ Exposure and the consumer use scenario is an essential first 'module', making use of 'expert assessments' and tools and consumer use data to determine whether the exposure is likely to be above the determined Threshold of Toxicological Concern; and then using toxicokinetics/toxicodynamics approaches (i.e. ADME) to assess whether the route of exposure leads to a relevant internal exposure and to indicate likely target organs for the parent substance and its metabolites. The predicted target organ(s) can be a focus for full assessment.

➠ Other information on the assessed substance can be incorporated in a flexible manner into the conceptual framework with the aim of defining relevant Adverse Outcome Pathways; notably 'read-across' information and other indicators of hazard such as structural alerts and '-omics' type 'fingerprint' information.

➠ 'General' chemicals and cosmetic ingredients may have multiple modes-of-actions across a narrow dose range, i.e. their perturbations will be non-selective rather than targeted to a particular biological mechanism (in contrast to drugs that tend to have a 'dominant' mode-of-action). Hence any approach should aim to define early in the process which scenario is relevant for the compound being assessed perhaps with a 'simplified' 'screening level' assessment. From this a decision can be made whether a protection goal driven by one specific Adverse Outcome Pathway, or one defined at an earlier casual mechanistic point in a Pathway not linked to any specific organ endpoint, can be used for assessment, or whether it is more appropriate to use the most sensitive point of departure.

➠ It is clear that the effects on organs can be assessed by several modes-of-action/Adverse Outcome Pathways and that the work on defining these is still at an early stage and not complete. At the meeting the **SEURAT-1** Safety Assessment Working Group focussed on liver toxicity (fibrosis) as the most advanced mode-of-action available and work will progress on how this can be utilised in the case study for strengthening a read across driven assessment. As future modes-of-action are defined and the data that is made available to populate the dose responses of the underpinning causal events in the mode-of-action, priority will be given to those where the likelihood of success in deemed greatest in completing examples of how to do non-animal safety decisions that can be shared with regulatory and industry bodies.

➠ Each Adverse Outcome Pathway begins with a Molecular Initiating Event and proceeds *via* a series of Intermediate Events, perhaps linked in a network and not in a simple sequence, to the adverse outcome. Some modes-of-action/ Adverse Outcome Pathways are already developed or in development (e.g. by the OECD), but it is accepted that there will be knowledge gaps. Some Adverse Outcome Pathways may be incomplete, but partial knowledge is still useful within the conceptual framework.

➠ Each 'block' within an Adverse Outcome Pathways (i.e. the Molecular Initiating Event and the Intermediate Events) are assessed using a Weight of Evidence approach, incorporating all relevant existing information (e.g. from 'classical' tests, QSARs and predictions etc). Each block can be 'populated' by a combination of *in vitro* assays (omics data, HPT data etc) and *in silico*

predictions to give a battery of tools. Existing tools can be included (from other 'new approach' programmes and academic research), but many will need to be added from the **SEURAT-1** Research Initiative. It is accepted that there will be knowledge gaps for many blocks. These assays/tools should be designed to be 'fit for purpose' to assess this particular biological property and not necessarily focussed on prediction of the complete protection goal as such they should not be 'over engineered', i.e. only an appropriate degree of technical validation would be necessary. In addressing the dose response kinetics of the assays it is clear that the uncertainty in the *in vitro* to *in vivo* extrapolations will need to be addressed.

➠ Uncertainty and incomplete knowledge in each Adverse Outcome Pathway assessment should be considered and addressed. In practice this may well mean assessing all the blocks in an Adverse Outcome Pathway even when one earlier in the pathway is 'negative'. Uncertainty at the framework level in the overall assessment should be considered and addressed. This could be done by a simple testing of sensitivity to different equivocal 'decisions' by testing the overall outcome if a different decision is taken at these 'nodes'. One approach through the use of different exposure scenarios should enable a decision to be made of regions of safety versus adversity. However the approach will also have to show applicability across different compounds, over and above the initial gold compounds used, to highlight relevance to cosmetic-type chemicals and ensure a generalised framework rather than a compound-specific mechanistic assessment.

➠ Understanding inter-individual and intra-individual differences and how they affect specific events in the pathway, and consequently alter dose-response relationships, might need to be integrated and made transparent within the overall assessment to incorporate the uncertainty driven by population variability.

➠ The overall outcome is anticipated to be robust as it is not based on single pieces of evidence.

➠ New knowledge on Adverse Outcome Pathways and new assays and prediction tools can be incorporated into the conceptual framework, i.e. it is flexible and will evolve and improve over time.

➠ The predictions from the conceptual framework should be validated as 'fit for purpose' for the particular regulatory purpose. The first step could be through discussions on the framework internationally in a regulatory forum to build consensus, leading to eventual adoption.

An indicative diagrammatic representation of this emerging conceptual framework is shown in *Figure 4.73*.

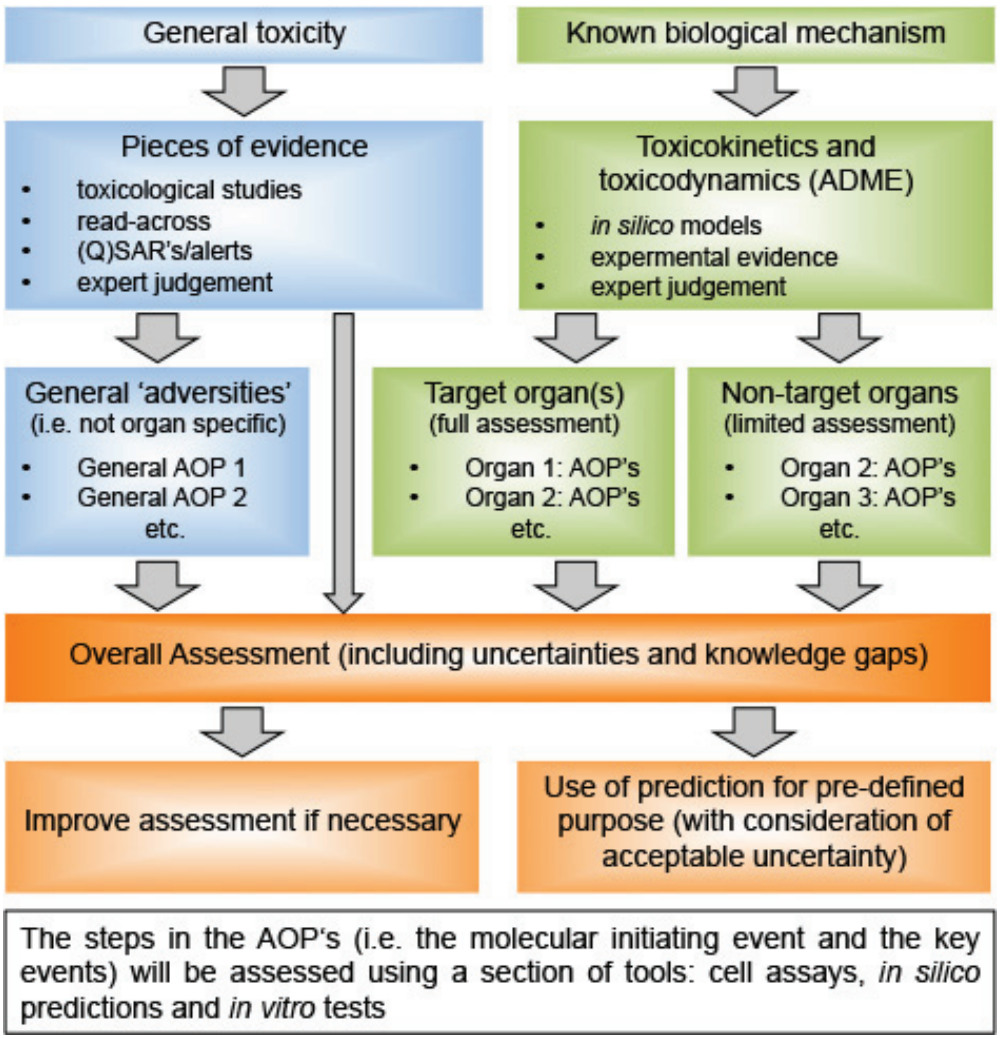


Figure 4.73 *Key elements of a conceptual framework for a rational integrated assessment strategy.*

Another way to picture the framework is as a flexible workflow that is adapted for the particular case (*Figure 4.74*). It should be noted that the 'downstream' elements are not fully elaborated, i.e. how the information is 'pulled together' to give an overall assessment'; these are issues that will become clearer as work proceeds.

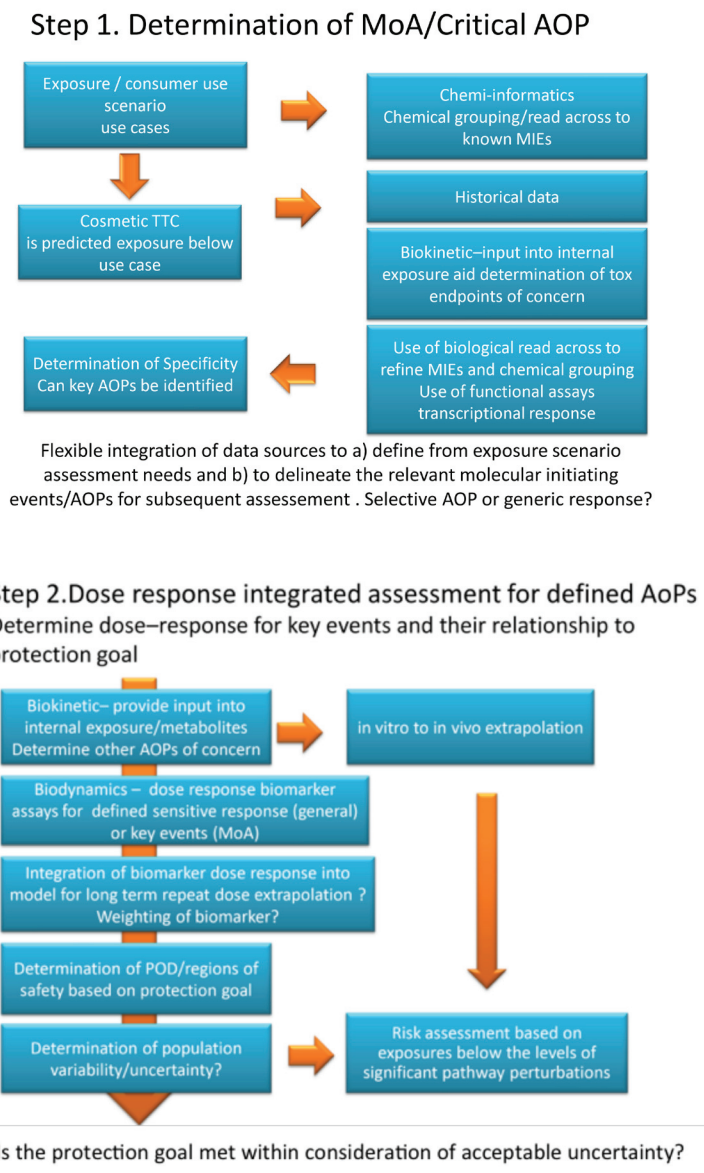


Figure 4.74 *Workflow for the integrated assessment strategy determining in a first step the AOP's and in a second step the dose-response relationships for these defined AOP's.*

The idea of an integrated assessment framework and the two case studies illustrating its use as a demonstration of proof of concept was a theme at the third **SEURAT-1** Annual Meeting in Lisbon on 6–7 March 2013. This was to enable researchers to be fully informed of the 'bigger picture' of **SEURAT-1** and also to communicate practical needs for testing that will contribute to the case studies.

4.10.9 Other Workshops

In addition to the Working Group meetings, other workshops were organised to address specific needs of the **SEURAT-1** Research Initiative projects. The intention was to hold high-level discussions on open questions and provide suggestions for future activities. In principle, the workshops were intended as a starting point for collaborations between cluster projects of the **SEURAT-1** Research Initiative.

The *HeMiBio* joint meeting with *SCR&Tox*, NOTOX and DETECTIVE on Bioreactors and Genetic Engineering of cells

The joint workshop of four **SEURAT-1** cluster projects took place at the University of Gent Culture and Convention Center Het Pand in Gent, Belgium, on 26 June 2012. This was the first joint meeting about the bioreactors and cell systems used by the four projects, and the aim was to connect researchers, facilitate sharing of information and build synergies concerning the bioreactors and cell types used in guaranteeing efficient progress towards the overall **SEURAT-1** objectives.

The overall objectives of the meeting were to identify synergies regarding (i) the different 3D bioreactor systems used for hepatocyte differentiation and culturing for long-term toxicity studies in the projects; and (ii) the genetic engineering of cells to be used in the bioreactors. Confidential information was exchanged through presentations of Principal Investigators from the different projects reporting on progress with respect to the above-mentioned areas.

Regarding the use of bioreactors, the discussion confirmed that the bioreactor developed in *HeMiBio* is indeed complementary to the one used by *SCR&Tox* and NOTOX. Regarding the types of cells used, several questions were put forward and further discussed in SEP meetings and within the **SEURAT-1** Stem Cell Working Group. Most importantly, the group discussed which characteristics the cells should have in order to be useful for long-term toxicity testing (i.e., the relevance of the different types of cells). Another issue considered was how to avoid duplication of work regarding genetic engineering of cells; as a solution, participants from the different projects agreed to share a list of genes (molecular sensors) targeted for genetic engineering.

Overall, this meeting stimulated fruitful discussions between the projects, which were also continued at the third **SEURAT-1** Annual Meeting in March 2013. A follow-up joint meeting on the same topics is planned for September 2013.

SCR&Tox: Assay Development Preparatory Meeting (written by Vania Rosas)

On 7 September 2012, the *SCR&Tox* consortium had a meeting in Ispra, Italy, (coupled with the six-monthly ex-comm meeting) in conjunction with COACH members. This meeting was held to discuss the selection of a relevant model – including compound(s), affected toxicity pathway(s) and relevant cell phenotype(s) – for further testing, first within the *SCR&Tox* consortium and further by the **SEURAT-1** Research Initiative. The second part of the *SCR&Tox* programme (starting in the 30th month, i.e. on 1 July 2013) required criteria to be defined and then a model selected that could be implemented according to the biological and technological resources developed by the first part of the *SCR&Tox* programme. This assay development preparatory meeting was the first step towards the selection of an *in vitro* stem cell-based assay, as a test system for promoting predictive toxicology at an industrial scale within the framework of repeated dose toxicity.

The *SCR&Tox* consortium members had a follow-up meeting in Paris on 7 June 2013, where a decision was made on the *in vitro* stem cell-based assay for predictive toxicology to be taken forward. This case study has been submitted for further consideration to COACH and the SEP of the **SEURAT-1** Research Initiative.

4.11 Training and Outreach

The COACH Team

4.11.1 Training Activities

Introduction

As outlined in the previous **SEURAT-1** Annual Reports, training is one of the essential parts of **SEURAT-1** outreach activities. Since the work programme of each of the six individual research projects had been defined independently, and each consortium had defined its own approach to addressing identified training needs, a common **SEURAT-1** training strategy has been developed through the initiative of COACH. A cluster-level training network has recently been set up and a common training programme defined.

In 2011 COACH began to homogenise the training activities at the cluster level by harmonisation of the cluster training plan. In order to coordinate the common training programme, COACH invited the research projects to set up a dedicated task force (**SEURAT-1** Training Task Force – STTF) composed of all seven projects' representatives for the training activities. In 2012 the implementation of the agreed programme started, with a cluster-level training event, the **SEURAT-1** Summer School 2012. The STTF also agreed that in one year's time (i.e., 2013) the individual research projects should have the opportunity to implement their own training programmes, focusing on internal training needs within their project and without overlap from the central **SEURAT-1** training activities.

Although no central training activity is planned for 2013, the cluster's third year plays a crucial role in crystallising the cluster training strategy and preparing the organisation of training activities for 2014. The STTF analysed the participants' feedback from the **SEURAT-1** Summer School 2012, allowing identification of the main shortcomings and how to address them. By defining the explicit *lessons learned,* **SEURAT-1** finalised the first cycle of its training strategy, as illustrated in the following *Figure 4.75*.

***Figure 4.75 SEURAT-1** training activities, from harmonisation to lessons learned.*

SEURAT-1 Summer School 2012

Looking back to 2012, the first **SEURAT-1** Summer School, held from 4–8 June 2012 at the Instituto de Biologia Experimental e Tecnológica (IBET), in Oeiras, Portugal, was key to supporting the integration of the young researchers, so as to increase efficiency as much as possible in their on-going research and development work. Close to 100 young fellows from **SEURAT-1** partner and external organisations, renowned speakers and other high-level scientists interested in this field of research came to Lisbon and participated in a very rich four-day programme of lectures and practical (mainly computer-based) sessions[1]. Some impressions from this first **SEURAT-1** Summer School are given in *Figure 4.76* and *Figure 4.77*, respectively.

The objectives of this first cluster-level summer school were to:

- spread knowledge related to **SEURAT-1** research areas within and beyond the cluster;
- provide an opportunity for the research fellows to meet colleagues from the other research groups, present and discuss their work, and to follow courses given by leading experts;
- create synergies and strengthen collaboration within the cluster.

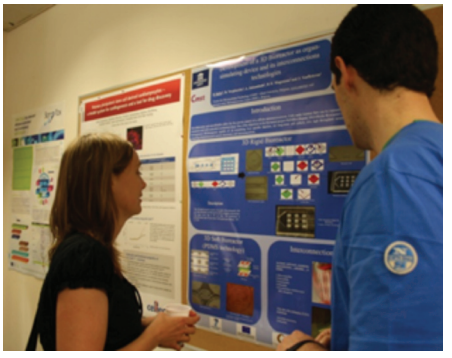


Figure 4.76 *Poster session during the summer school.*

The summer school programme featured a mix of sessions and events:

- Lectures were given by renowned scientists from Cosmetics Europe, ECHA, IBET or other related initiatives, such as the ESNATS project (http://www.esnats.eu/).
- Close to 30 presentations were given by the **SEURAT-1** principal investigators as well as by young researchers.

1. Please refer to the second volume of the **SEURAT-1** Annual Report for more information about the summer school programme (http://www.seurat-1.eu/pages/library/seurat-1-annual-report.php).

➠ Practical hands-on workshops were organised (mostly in the form of computer sessions) in small groups, allowing individual teaching.

➠ Several poster sessions triggered lively discussions between the students but also gave an opportunity for the students to present their work to the invited experts and SEP members.

➠ Exclusive visits to IBET's Animal Cell Technology Laboratories, and their pilot and current Good Manufacturing Practice (cGMP) manufacturing plants.

➠ A social event in form of a guided tour in Lisbon was organised, giving space to the participants to interact and network. Lunch and dinner was organised in the same spirit; the participants could enjoy sunny weather while discussing (in)formally with each other.

Figure 4.77 SEURAT-1 *Summer School 2012 exchange and interactions.*

The feedback received during the summer school was extremely positive. The participants very much appreciated the quality of the presentations and the practical organisation. The event was also found to be a suitable environment for scientific exchanges and the creation of long-lasting links between groups working in related research domains. As a follow-up to the summer school, an anonymous survey was established and circulated, composed of seven questions regarding the programme, the organisation, the location and the possibility for interaction. The results of the survey reflected the overall satisfaction of the participants with the training event, its organisation by the COACH Office, the programme and the facilities. This result endorses the positive reactions received during the event itself.

Nevertheless, the feedback questionnaire also revealed a couple of points where improvements should be considered for the next event organisation, concerning the venue, the programme and the session attendance. The shortcomings were presented and discussed during a special STTF session organised during the Annual Meeting in March 2013. Based on the discussions, the COACH Office identified three main *lessons learned* to be implemented for the next training activities:

➠ It would be preferable to hold the next summer school in a more central location so that the travel costs and time of the participants are reduced;

➠ The research project partners should be encouraged to propose more practical sessions. Long theoretical conference-type sessions should be avoided;

➠ The cluster should increase the visibility of its training activities and attractiveness for **SEURAT-1** and non-SEURAT researchers, and thus ensure higher attendance of the training event.

SEURAT-1 Summer School 2014

One global aim of **SEURAT-1** is to promote and facilitate exchanges with other related initiatives. The organisation of **SEURAT-1** training activities should also keep this aim in mind. The first **SEURAT-1** Summer School was, therefore, also opened to trainees and speakers from outside the **SEURAT-1** Research Initiative. In 2014 there will be an attempt to express the spirit of collaboration further. At the time of writing, **SEURAT-1** and the European Society of Toxicology *in Vitro* (ESTIV) are considering whether to organise a joint training event in summer 2014. Both initiatives are currently discussing how to join their forces in the most efficient way and how best to organise an attractive '**SEURAT-1** and ESTIV Joint Summer School 2014'. Those interested in participating should monitor the 'News and Events' section on the **SEURAT-1** public website (http://www.seurat-1.eu/).

Training Activities

As mentioned above, because no cluster-level central training activity was foreseen for 2013, the year was dedicated to individual project-level training events according to internal needs, in particular hands-on lab training. The following training events took place in 2013:

HeMiBio Winter School: This one-day course was intended to help non-toxicologists become familiar with the field of toxicology and risk assessment, and was held on 16 January 2013 in Barcelona, Spain. Focus was put on basic toxicology as well as on four topics specifically tailored for the **SEURAT-1** research programme, including: (i) the safety evaluation of cosmetic ingredients; (ii) mechanisms and types of liver toxicity; (iii) biomarkers of (liver) toxicity; and (iv) *in vivo* extrapolation of *in vitro* toxicity data.

HeMiBio Summer School: Planned as an hands-on training event, this summer school entitled 'Practical Concepts of *in vitro* and *in silico* Toxicology' was held on 4–6 June 2013 in Brussels, Belgium. Following the winter school, which was focused on teaching theoretical concepts of

toxicology and risk assessment, this *HeMiBio* workshop equipped participants with practical skills of *in vitro* and *in silico* toxicology. On the first day of the workshop, the standard operating procedures as well as the theoretical background of the different *in vitro* methods involved were presented, followed by their practical demonstration. Focus was to put on methods for evaluating the functionality (e.g., cytochrome P450 activity, drug transporter activity, albumin secretion and urea synthesis) and the toxicological response (e.g., caspase 3 activity, lactate dehydrogenase release, MTT assay, *in situ* staining of cell death markers, Sudan Red III staining, LipidTOX assay and reactive oxygen species measurement) of cultured primary hepatocytes. During the second day, the participants were given the opportunity to gain hands-on experience with the aforementioned techniques and to discuss strategies for their application to other cell types (e.g., hepatocyte-like cells differentiated from stem cells) and experimental settings (e.g., 96-well plate formats). At the start of the third day, a number of lectures were given dealing with quantitative *in vitro–in vivo* extrapolations and dose metrics in *in vitro* assays. The workshop was closed with a hands-on computer session on physiologically based pharmacokinetic modelling.

DETECTIVE Summer School: The DETECTIVE summer school took place in Slano, Croatia, on 10–14 June 2013. The aim was to encourage mutual training and exchange of views and knowledge between all DETECTIVE partners. The programme contained talks and hands-on training by DETECTIVE and other invited **SEURAT-1** project partners, keynote lectures by external speakers and a poster session.

4.11.2 Workshops

Within **SEURAT-1** a number of workshops were held in the second year and are currently being organised within the Working Groups (see chapter 4.10.2–4.10.8). In addition to these, several workshops took place outside the Working Group activities to address specific aspects of repeated dose systemic toxicity. Respective reports are given in chapter 4.10.9.

4.11.3 Conferences

SEURAT-1 had a unique opportunity to present its latest results at the prestigious international Euroscience Open Forum (ESOF), which took place in Dublin on 14 July 2012. At this occasion, **SEURAT-1** organised a press conference and was represented at a session entitled ‘Are there realistic alternatives to animal testing?’. Mark Cronin from the Liverpool John Moores University, the coordinator of the COSMOS project, and Maurice Whelan from the European Commission Joint Research Centre, a consortium member of the COACH project, were among the speakers.

In addition, the second volume of the **SEURAT-1** Annual Report was officially launched during the event (*Figure 4.78*), which considerably increased interest in the publication, noticeable especially through the increase of hits on the public website (see the subsequent chapter on public website).

***Figure 4.78 SEURAT-1** Annual Report launch at ESOF 2012.*

The press conference also led to articles in several journals:

- Clive Cookson, Synthetic life: the revolution begins (*The Financial Times*, 27 July 2012);
- Michelle Yeomans, Ambitious consortium reports considerable progress in animal testing area at EU forum (*Cosmetics Design-Europe*, 18 July 18 2012);
- Ronan McGreevy, Chemical testing without animals 'close' (*The Irish Times*, 16 July 2012);
- EU Research Program **SEURAT-1** Declares Progress Towards Animal Free Testing (*Special Chem*, 20 July 2012);
- Cosmetics testing – the way forward in Europe (*Animal Defenders International*, 19 July 2012);
- Pharma And Biotech, Cosmetics: pioneering the way towards animal free testing (*Scientist Live*, 20 July 20 2012).

The **SEURAT-1** Research Initiative was further represented at a number of international conferences in the last year, as summarised in *Table 4.19*.

Table 4.19 *Presence of the* ***SEURAT-1*** *Research Initiative in international conferences and workshops.*

Conference	Date	Place	Contribution	Project
National Academy Science Symposium on Systems Toxicology in Risk Assessment	14–15 June 2012	Washington DC, USA	Presentation of **SEURAT-1**	COACH
15th International Workshop on Quantitative Structure-Activity Relationships (QSAR 2012)	18–22 June 2012	Tallinn, Estonia	Presentations and posters	COSMOS
Invited seminar, Safety and Environmental Assurance Centre, Unilever	4 July 2012	Colworth, UK	Presentation of **SEURAT-1**	COACH
Federal Institute for Risk Assessment seminar	8 August 2012	Berlin, Germany	Distribution of the 2nd **SEURAT-1** Annual Report	COACH
13th IEEE International Conference on Information Reuse and Integration	8–10 August 2012	Las Vegas, USA	Presentation	COSMOS
19th EuroQSAR	26–30 August 2012	Vienna, Austria	Presentations and posters	COSMOS
Scientific Advisory Committee on Alternative Toxicological Methods - SACATM	5–6 September 2012	New York, USA	Distribution of the 2nd **SEURAT-1** Annual Report	COACH
The 3rd International Lhasa Symposium: New Horizons in Toxicity Prediction	5–6 September 2012	Cambridge, UK	Presentations and posters	COSMOS
17th European Congress on Alternatives to Animal Testing, 14th Annual Congress of EUSAAT	5–8 September 2012	Linz, Austria	Distribution of the 2nd **SEURAT-1** Annual Report	COACH
Meeting of the Slovenian Chemical Society	12–14 September 2012	Portoroz, Slovenia	Presentations and posters	COSMOS
CADASTER Workshop on the Development and Application of QSAR Models	7–9 October 2012	Munich, Germany	Presentations	COSMOS
European Parliament Event on Horizon 2020 and Safety Assessment	10 October 2012	Brussels, Belgium	Presentation of **SEURAT-1**	COACH
27th International Federation of Societies of Cosmetic Chemists Congress (IFSCC)	15–18 October 2012	Johannesburg, South Africa	Presentation, distribution of the 2nd **SEURAT-1** Annual Report	COSMOS
European Society of Toxicology *In Vitro* 2012 Annual Meeting (ESTIV)	16–19 October 2012	Lisbon, Portugal	Presentations and posters, distribution of the 2nd **SEURAT-1** Annual Report	COACH, COSMOS, *HeMiBio*, DETECTIVE

CAAT Conference	25 October 2012	Konstanz, Germany	Invited presentations, distribution of the 2nd **SEURAT-1** Annual Report	*HeMiBio,* NOTOX, COACH
Annual Meeting of the American College of Toxicology	4–7 November 2012	Orlando, USA	Presentations and posters, distribution of the 2nd **SEURAT-1** Annual Report	COSMOS
21st Conference on Current Trends in Computational Chemistry (CCTCC)	9–10 November 2012	Jackson, USA	Presentations and posters	COSMOS
The EPAA Annual Conference - Global Cooperation on alternatives (3Rs) to animal testing	16 November 2012	Brussels, Belgium	Presentation, poster, distribution of the 2nd **SEURAT-1** Annual Report	COACH COSMOS
1st Latin American Congress on Alternative Methods to Animal Use in the Education, Research and Industry (COLAMA 2012)	25–29 November 2012	Rio de Janeiro, Brazil	Presentation and distribution of USB sticks with both **SEURAT-1** Annual Reports	COACH
International Workshop on Current and future prospects of alternative methods for cosmetics safety testing	29–20 November 2012	Brasilia DC, Brazil	Presentation and distribution of **SEURAT-1** USB stick with both Annual Reports	COACH
Invited seminar at the National Toxicology Programme, NIEHS	29 January 2013	North Carolina, USA	Presentation of **SEURAT-1**	COACH
Tox21 Annual Meeting, US EPA	31 January 2013	Washington DC, USA	Presentation of **SEURAT-1**	COACH
The European Parliament Intergroup on the Welfare and Conservation of Animals (291st Meeting)	7 February 2013	Strasbourg, France	Presentation of **SEURAT-1**	COACH
European Association for the Study of the Liver Monothematic Conference: Systems Biology of the Liver	21–23 February 2013	Luxembourg	Presentation	NOTOX
52nd Annual Meeting of the Society of Toxicology (SOT)	10–14 March 2013	San Antonio, Texas	Presentations, poster	COSMOS
Annual Meeting of the European Association for the Study of the Liver	24–28 April 2013	Amsterdam, The Netherlands	Presentations, poster	*HeMiBio*, NOTOX
Systems Toxicology 2013 – From Basic Research to Human Risk Assessment	28 April – 1 May 2013	Ascona, Switzerland	Presentation of **SEURAT-1**	COACH
Meeting of the Advisory Group on Molecular Screening and Toxicogenomics, OECD	14 May 2013	Paris, France	Presentation of **SEURAT-1**	COACH
In Vitro Testing Industrial Platform (IVTIP) Spring 2013 meeting	16–17 May 2013	Southampton, UK	Presentation of **SEURAT-1**	COACH
US FDA Workshop: Scientific Roadmap for the Future of Animal-free Systemic Toxicity Testing	30–31 May 2013	College Park, USA	Presentation of **SEURAT-1**	COACH

The importance of the international conferences for **SEURAT-1** can be conclusively demonstrated when analysing the public website statistics. For example, the International Pharmaceutical Science Congress 2013 (CIFARP2013) to be held in November 2013 in Brazil, at which Mark Cronin (COSMOS) will give a talk about **SEURAT-1**, encouraged a number of new visitors to find more information about **SEURAT-1** by visiting the website. The following graphic (*Figure 4.79*) shows that Ribeirão Preto, the venue of CIFARP2013, has become the city with the most visits to the website in the period from 1 January 2013 to 4 June 2013 (together with Paris).

	City	Visits	% Visits
1.	Ribeirao Preto	235	6.84%
2.	Paris	235	6.84%
3.	City of Brussels	173	5.03%
4.	Ispra	119	3.46%
5.	London	108	3.14%

***Figure 4.79 SEURAT-1** conferences impact on **SEURAT-1** visibility (source: Google Analytics).*

4.11.4 Prizes and Awards

JRC team wins first Lush Science Prize for non-animal safety testing: In November 2012 at an awards ceremony in London, a JRC team received the first Lush Science Prize for their pioneering work on non-animal chemical safety testing. The new annual Lush Science Prize, a joint project between the global handmade cosmetics company Lush and *Ethical Consumer* magazine, is designed to bring forward the date when ingredients for cosmetics and household products are no longer tested on animals. A key part of the prize is to reward 'outstanding contributions' to '21st Century Toxicology' – a new approach to chemical testing. Using recent advances in genetics and computer science it aims at improving the prediction of the potential toxicity of a substance without animal testing.

The winning team designed and demonstrated a high-throughput screening system to categorise chemicals based on their potential to cause liver toxicity. The approach uses in-depth knowledge of toxicological processes to identify key biological events that can be captured in a suite of specially tailored *in vitro* assays. Professor Maurice Whelan, head of the Systems Toxicology Unit and the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM), expressed his appreciation for the recognition this award brings and stated that the prize money of £50,000 will be used to advance work in this area.

COSMOS: Winning of the EPAA Poster Prize: Liverpool John Moores University from COSMOS won the EPAA Poster Award for 2012, for the poster 'An International Cooperative Project Developing Computational Models for Repeated Dose Toxicity' by Dr Andrea-Nicole Richarz. The EPAA Poster Award is given annually for a poster presented by not-for-profit organisations during the EPAA Annual Conference.

4.11.5 SEURAT-1 Public Website

The **SEURAT-1** public website (http://www.seurat-1.eu/) went online in 2011 and is still an essential information channel for people interested in **SEURAT-1** research and related aspects. During late 2012 and early 2013 the website went through a number of updates:

- The content of the website has been updated to reflect the **SEURAT-1** strategy and its concrete implementation in the course of the cluster's life.
- A special page intended for laypeople (e.g., students, teachers, school-aged children) has been created as a part of the new dissemination activities. This section clarifies in a very simple way the principal issues linked to the toxicology research as well as the role of **SEURAT-1**.
- Information about the Working Groups has been added, as they play an important role in the cluster and should have a good visibility outside of the cluster.
- The second **SEURAT-1** Annual Report has been made available for download.
- The 'News' section is being updated in a timely fashion; the projects are progressively providing more cluster project news to the COACH Office.
- Several job offers were published, the calendar of the **SEURAT-1** events has been updated, and the 'Who is who' section was completed with new profiles of people involved in the **SEURAT-1** Research Initiative.

With regards to the number of visitors, the following *Figure 4.80* and *Figure 4.81* give a summary overview of the visits to the website. The period after the launch of the Annual Report and after the **SEURAT-1** Annual Meeting remain the two busiest events for the public website.

	Country / Territory	Visits	% Visits
1.	United States	70	14.89%
2.	Brazil	57	12.13%
3.	United Kingdom	55	11.70%
4.	France	48	10.21%
5.	Germany	46	9.79%
6.	Italy	32	6.81%
7.	Belgium	29	6.17%
8.	Japan	19	4.04%
9.	Switzerland	10	2.13%
10.	Lithuania	10	2.13%

***Figure 4.80 SEURAT-1** conferences impact on **SEURAT-1** visibility (source: Google Analytics).*

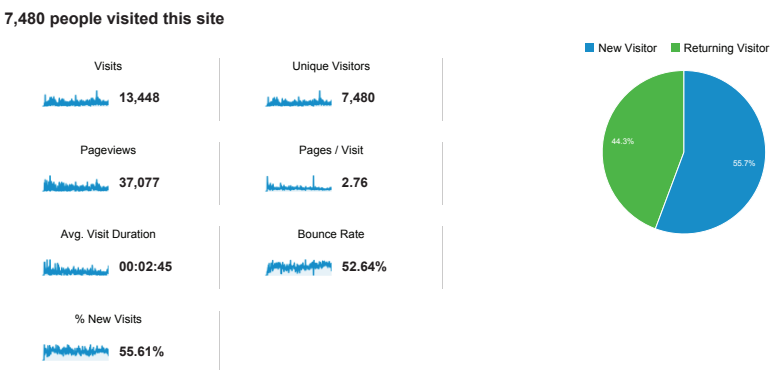


***Figure 4.81** Total number of visits to **SEURAT-1** website since July 2011 (source: Google Analytics).*

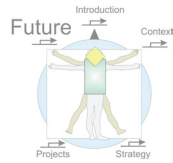
4.11.6 Other Dissemination Material

Most of the **SEURAT-1** dissemination material was created in the first year and is still being used, such as leaflets, posters and PowerPoint presentations. These means of communication have been updated according to the new developments within the cluster.

USB stick: A new dissemination means has been introduced in 2012 in the form of a USB stick (*Figure 4.82*). With the increasing number of important documents to be distributed either internally within the **SEURAT-1** members or externally to various conference participants, the COACH Office decided to introduce the **SEURAT-1** USB stick. Using the USB stick helps to efficiently disseminate existing **SEURAT-1** material, especially the Annual Reports, not only during the cluster Annual Meetings but also to those conferences where the shipment of printed documents (and in particular the Annual Reports) would create significant logistic constraints and represent a heavy financial burden. In 2012 a complete set of **SEURAT-1** documents, including the Annual Reports (volumes 1 and 2), were distributed on **SEURAT-1** USB sticks during the COLAMA 2012 event in Rio de Janeiro, Brazil.

Figure 4.82 *The* ***SEURAT-1*** *USB stick.*

Video material (Documentary): The NOTOX project has produced an eight-minute film, in which the main aims of the project as well as the people behind it are introduced, and the challenges of developing validated alternative testing methods for long-term systemic toxicity are explained. Featuring Dr Ruxandra Draghia-Akli, head of the Health Directorate General of the European Commission, the film showcases the efforts being made within the NOTOX project as part of the European research cluster **SEURAT-1** to find safe solutions to replace animal testing for safety assessment of cosmetic and hygienic products. The film underlines how leading academic experts from various research fields, along with representatives from small- and medium-sized enterprises, collaborate successfully to reach this goal. Hard copies of the film can be sent to interested stakeholders upon request (contact via NOTOX coordinator Elmar Heinzle). The NOTOX film is also available on the project website: http://www.notox-sb.eu/film



5 PREPARING FOR THE FUTURE

'International cooperation on 3Rs is 'not nice to have', it's 'must have'.'

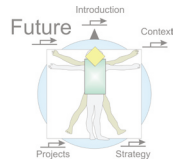
Jacqueline Minor, European Commission, Director of Consumer Affairs at DG SANCO[1].

1. In: Full Report of the 8th EPAA Conference 2012 (http://ec.europa.eu/enterprise/epaa/3_events/ann-conf-2012/conf-2012-report.pdf, accessed 10 June 2013)

5.1 Introduction

Taking into account the complexity of the problems to be solved and the broadness of the expertise needed to address the underlying scientific questions, the **SEURAT-1** Research Initiative will not be able to finalise the necessary work for full replacement of animal testing in the area of repeated dose systemic toxicity within the next years. Indeed, moving from animal testing to mode-of-action based *in vitro* assays for improved human safety assessment will require the combined efforts of European and other international activities. The **SEURAT-1** Research Initiative is operating in a very dynamic field of research, and a number of related research projects in different parts of the world are active in parallel. This chapter will provide an overview about these parallel research programmes by presenting short descriptions as a basis for the identification of complementary activities and, most importantly, possible future collaborations.

The aim is, in fact, to establish close international cooperation over the course of **SEURAT-1**, and to advance scientific progress in this field of research by using the synergy of a collaborative approach, which is yet to be fully developed. This will provide the basis for the identification of gaps of knowledge that need to be addressed in the future. The **SEURAT-1** Working Groups play a major role in this context, as the 'natural' contact point between **SEURAT-1** and the related international projects is the organisation of workshops. As outlined in the Terms of Reference for the working groups (see second volume of this Annual Report), the working groups are charged with dealing with specific research questions and problems originating from project activities with the aim of finding common solutions on the cluster level; that is, the reinforcement of **SEURAT-1** internal cooperation (see chapter 4.10). Furthermore, the working groups also have a think tank aspect to encourage creativity and capture external expert views, with the aim of achieving a large and multidisciplinary perspective; it is exactly this aspect that provides the starting point for exchange activities between **SEURAT-1** and other, related international projects in the field. Some key areas for such collaboration were already identified during workshops in the past year and this is also highlighted in the summary reports in the following sections. The efforts of setting up international collaborations culminated in a workshop dedicated to the identification of common interests between **SEURAT-1** and the related initiative in the USA, Tox21, as a basis for future exchange activities, and the outline of this workshop is given in a separate section. Considering the elements of the different approaches in the USA and Europe and taking into account that at this time it is midterm of the **SEURAT-1** Research Initiative, the demand for outlining the continuation of the SEURAT work programme beyond **SEURAT-1** is increasing. Therefore, some thoughts about elements of a possible second SEURAT phase complete this third **SEURAT-1** Annual Report.



5.2 Related International Activities

The COACH Team

The following sections provide an overview of parallel research activities as a basis for future collaborations between **SEURAT-1** and other consortia. The descriptions have been kept very brief and were, in parts, taken directly from published descriptions of corresponding projects. The sources used are given at the end of each project summary (in general, this refers to a public webpage). Only currently running activities (research projects as well as institutions) are considered in this compilation.

5.2.1 European Activities

EU FP7: 7th Framework Programme of the European Union represented by the European Commission

The Health Work Programme 2013 has an indicative budget of around EUR 840 million to cover many health issues. Most importantly for the **SEURAT-1** Research Initiative in the last year was the publication of a call for proposals entitled 'Modelling toxic responses in case studies for predictive human safety assessment', which was published under the HEALTH Theme on 10 July 2012 (Call Identifier: HEALTH.2013.1.3-1 within FP7-HEALTH-2013-INNOVATION-1). The main objective of this topic is to exploit the recent advances in computational chemistry and systems biology in case studies, in order to provide the basis for innovative approaches to predictive human safety assessments. Integrated research should be undertaken that:

- Considers modelling transport and interactions from molecular to cellular/ organelle levels;
- Integrates with *in vitro* experimentation designed specifically to inform this modelling activity;
- Couples directly to systems modelling from cellular to organ level;
- Takes into account the mechanistic understandings of toxic responses in specific organs;
- Uses existing and appropriate infrastructure for computation data basing and sharing.

In addition to the development of a comprehensive strategy and research concept, the following issues should be addressed at either the theoretical or the experimental level:

- Identification of metabolites (and metabolites of metabolites) and their

reactivity, through a combination of computational chemistry, *in vitro* experimentation and enzyme expression profiling;

➠ Identification of the proteins and potentially other intracellular targets affected by each metabolite, through computational chemistry and *in vitro* work;

➠ Identification of the pathways affected by these proteins, through *in vitro* cell assays and systems biology;

➠ Identification of cell functions affected by these pathways, by defining the boundaries of normal function and understanding the physiology and systems biology.

More information: http://cordis.europa.eu/fp7/health/

Envisaged Cooperation:

Many of the objectives are complementary with **SEURAT-1** and, thus, the relationships with the **SEURAT-1** Research Initiative are obvious. A close cooperation with the successful consortium is foreseen. The selection follows a two-stage procedure: the deadline for the first stage was on 3 October 2012 and the deadline for the second stage on 6 February 2013. The evaluation of all second-stage full proposals is finished and it is expected that the successful proposal will be identified soon.

AXLR8: Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development

AXLR8 is a coordination action funded within the European Commission's 7th Framework Programme under the HEALTH Theme. It has been established as a focal point for dialogue, collaboration and coordination among 3Rs ('Replacement, Reduction and Refinement of animal testing') research projects at national, European and international levels. The purpose of the project is to monitor the EU FP6/FP7-funded 3Rs research activities and related international initiatives, with the aim of accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment. AXLR8 will facilitate the swift progress to more mechanistic cell- and computer-based techniques. This overarching goal is divided into different specific objectives: (i) monitoring activities of new DG-RTD-funded FP6/FP7 3Rs research consortia and preparation, publication and dissemination of progress reports on an annual basis; (ii) organising annual workshops to monitor research progress and identifying gaps and needs in the FP6/FP7 programme on alternative testing strategies; (iii) promoting the establishment of a Scientific Panel (SP) as a platform for information exchange – this will comprise

representatives of EU-funded 3Rs research projects together with independent experts from EU corporate, governmental and academic sectors; (iv) gaining stakeholders and increasing public awareness of the outcomes of AXLR8 scientific workshops and safety assessment; (v) promoting viable and on-going engagement by European regulatory authorities in order to streamline regulatory acceptance procedures and to provide for the uptake of validated 3Rs methods, including a smooth transition to 21st-century systems, as they become available.

Scientific Coordinator: Horst Spielmann (Freie Universität Berlin, Germany)

More information: http://axlr8.eu

Existing Cooperation:

Maurice Whelan (Principal Investigator in the **SEURAT-1** project COACH) is a member of the above-mentioned Scientific Panel. Summary reports of **SEURAT-1** activities were presented at the occasion of the 2011 and 2012 AXLR8 workshops and are also part of the respective AXLR8 Annual Report.

Predict-IV: Profiling the toxicity of new drugs: a non animal-based approach integrating toxicodynamics and biokinetics

Predict-IV is a collaborative large-scale Integrated Project, which is funded within the European Commission's 7th Framework Programme (FP7) under the HEALTH Theme. Predict-IV started in 2008 and will run until 2013. Overall, 21 European participants of different scientific sectors (academia and industry) are working on this project. The overall goal is the development of new strategies for improved assessment of drug safety in the early stage of development and a late discovery phase.

The project is motivated by the existing deficit of preclinical toxicity testing approaches, which can be explained by both the lack of therapeutic efficiency and an unpredicted toxicity in animals and humans. New acquisitions in tissue and bioreactor technologies, molecular biology, toxicity modelling and bioinformatics are integrated in Predict-IV to improve and optimise cell culture systems for toxicity testing. Predict-IV will form a combination of classical *in vitro* toxicology and recent technologies, profiling and modelling tools in a systems biology approach. High quality standards on modelling and biostatical analysis will be used for analysis, evaluation and integration of data from *in vitro* experiments. Additionally, Predict-IV will highlight advances in '-omics' technologies and high-content imaging and therefore increase the probability for the early identification of toxic effects of pharmaceuticals.

Scientific Coordinator: Wolfgang Dekant (Universität Würzburg, Germany)

More information: http://www.predict-iv.toxi.uni-wuerzburg.de/

Existing collaboration:

Some members of the **SEURAT-1** consortium are also active in Predict-IV (Paul Jennings/ Innsbruck Medical University, Austria; Annette Kopp-Schneider/German Cancer Research Center, Heidelberg, Germany; Pilar Prieto/Joint Research Centre, Ispra, Italy). An interest shared by both consortia is the use and interpretation of '-omics' data for the identification of biomarkers for toxicity.

ESNATS: Embryonic stem cell-based novel alternative testing strategies

ESNATS is also funded within the European Commission's 7th Framework Programme (FP7) under the HEALTH Theme. It started in 2008 and will run until 2013. All in all, 27 European participants are involved in ESNATS. This project demonstrates a new type of platform for toxicity testing using the different advantages of embryonic stem cells (ESCs), especially human ESCs. Using these cell types, which are characterised by their self-renewal capacity, their pluripotency and the impact of ES-derived somatic and murine cells, the project aims to achieve three overall objectives: to accelerate drug development, to reduce related Research and Development costs and to propose a powerful alternative to animal tests. The achievement of these key objectives requires the examination of different ethical issues involved in the ESNATS scientific programme when working with ESCs. For this reason, the work of the ESNATS consortium is advised by the ethical organisation Edinethics Ltd in order to comply with the strict requirements specified by the European Commission for FP7 research programmes.

ESNATS is divided into four key research areas, covering the following complementary scientific aspects: (i) the sub-project entitled 'Reproductive Toxicity' will investigate the possible hazards of compounds to the reproductive cycle, i.e., impact on fertilisation, differentiation into gametes (male fertility) and early embryonic development; (ii) the sub-project 'Neurotoxicity' deals with the effects of compounds on neuronal development and viability (functionality); (iii) the sub-project 'ESC-based toxicogenomics and toxicoproteomics' focuses on the influence of compounds on gene expression and proteomics using *in vitro* test systems suitable for high-throughput methods; (iv) the sub-project 'Metabolism, Toxicokinetics and Modelling' concentrates on the development of physiologically based pharmacokinetic (PBPK) models using *in vitro* data.

Scientific Coordinator: Jürgen Hescheler (University of Cologne, Germany)

More information: http://www.esnats.eu

Existing collaboration:

Some members of the **SEURAT-1** consortium are also active in ESNATS (Jürgen Hescheler/

University of Cologne, Germany; Jan Hengstler/IFADO, Dortmund, Germany; Susanne Bremer-Hoffmann/Joint Research Centre, Ispra, Italy; André Schrattenholz/ProteoSys, Mainz, Germany; Vera Rogiers/Vrije Universiteit Brussel, Belgium). Mode-of-action descriptions of neurotoxicity are currently under discussion in **SEURAT-1**, and common interests between both consortia exist also in the fields of using '-omics' data for biomarker identification and the use of PBPK models for *in vitro* to *in vivo* extrapolation.

ChemScreen: Chemical substance *in vitro* / *in silico* screening system to predict human- and ecotoxicological effects

ChemScreen is a collaborative project, which is funded within the European Commission's 7th Framework Programme (FP7) under the Environment programme. The project started in 2010 and will run for four years. ChemScreen is a sister project of the US Environmental Protection Agency's National Center for Computational Toxicology (NCCT/STAR centre) and is therefore strongly linked to related projects in North America (Toxcast, Tox21; see project descriptions below). Nine project partners from five countries of the European Union are working together in ChemScreen with the overall goal of developing innovative, animal-free screening methods for the assessment of toxicological and ecotoxicological effects of chemicals in the field of reproductive toxicity.

Scientific Coordinator: Bart van der Burg (BioDetection Systems BV, Amsterdam, The Netherlands)

More information: http://chemscreen.eu/

diXa: Data Infrastructure for Chemical Safety

'Data Infrastructure for Chemical Safety' (diXa) is funded under the European Commission's 7th Framework Programme. The project started in October 2011 and will run until September 2014. The main objective of the diXa project is to further develop and adopt a robust and sustainable service infrastructure (e.g., data infrastructure and e-science environment) for harbouring multiplexed data sets, as produced by past, current and future EU research projects on developing non-animal tests for predicting chemical safety, in conjunction with other globally available chemical/toxicological databases and databases on molecular data of human disease. diXa focuses on networking activities for building a web-based, openly accessible and sustainable e-infrastructure for capturing toxicogenomic data, and for linking this to available databases holding chemico-/physico-/toxicological information, and to databases on molecular medicine, thus crossing traditional borders between scientific disciplines and reaching out to other research communities.

To advance data sharing with research communities, diXa ensures clear communication

channels with and delivers commonly agreed core service support to the toxicogenomic research community, by providing SOPs for seamless data sharing, and by offering quality assessments and newly developed tools and techniques for data management, all supported by hands-on training. Through its joint research initiative, by using data available from its data infrastructure, diXa will demonstrate the feasibility of its approach by performing cross-platform integrative statistical analyses, and cross-study meta-analyses, to create a systems model for predicting chemical-induced liver injury.

Scientific Coordinator: Jos Kleinjans (University of Maastricht, The Netherlands)

More information: http://www.dixa-fp7.eu/

Existing collaboration:

Clemens Wittwehr from the **SEURAT-1** consortium (Joint Research Centre, Ispra, Italy) is a partner in the diXa project.

IMI: Innovative Medicines Initiative

The Innovative Medicines Initiative Joint Undertaking (IMI JU) is a unique pan-European public–private partnership between the European Commission and the European Federation of Pharmaceutical Industries and Associations (EFPIA), driving collaboration between all relevant stakeholders, including large and small biopharmaceutical and healthcare companies, regulators, academia and patients. IMI's research projects that are selected for funding through open calls for proposals have to adhere to the four areas (the so-called Four Pillars) of the Strategic Research Agenda: (i) Predictivity of Safety Evaluation; (ii) Predictivity of Efficacy Evaluation; (iii) Knowledge Management; and (iv) Education and Training. In total, four calls for proposals were launched in 2012 (4th–8th Call 2012) and the details of the successful proposals from the 5th ('European Lead Factory') and 6th calls (two projects focusing on antibiotic development) were announced in February 2013. The topics of the 7th call are (i) Developing a framework for rapid assessment of vaccination benefit/risk in Europe; and (ii) Incorporating real-life clinical data into drug development. The most relevant for the **SEURAT-1** activities is the 8th call, which addresses the three following themes: (i) Combating Antibiotic Resistance; (ii) Developing an Aetiology-based Taxonomy for Human Diseases; and (iii) European Induced Pluripotent Stem Cell Bank. In the following we highlight some details from the latter theme, as it explicitly mentions the **SEURAT-1** Research Initiative as a potential partner for collaboration.

Rapid advances in iPS cell-related research will result in new approaches including efficacy and toxicity testing of new therapies using iPS cells differentiated from disease-relevant populations. It is, however, important to generate an iPS cell bank that consistently provides

research scientists with quality reagents generated under standardised validated or GLP conditions. The unique attribute of the envisaged European iPS cell bank will be the ability to provide patient-derived iPS cell cultures that are quality assured and established within defined timelines at scale to support academic researchers, private–public partnerships and industry for research, early drug discovery and safety assessment. Consequently, the focus of this new cell centre would be on iPS cell lines derived from carefully genotyped and phenotyped patients. The overall objectives of this project are:

- to identify key cohorts of patients that are useful for research purposes within the wider scientific community;
- to create a large single European iPS cell repository hosted in an appropriate facility;
- to generate a centre of scientific excellence for standardisation and optimisation in cryopreservation, retrieval and differentiation methods for iPS cell lines.

More information: http://www.imi.europa.eu/content/

Envisaged cooperation:

These topics are obviously closely related to projects within the **SEURAT-1** Research Initiative that are dealing with the generation of iPS cells for toxicity testing (i.e., *SCR&Tox* and *HeMiBio*). Furthermore, the **SEURAT-1** Stem Cell Working Group (see chapter 4.10.7) would be the ideal platform for establishing a collaboration with this IMI initiative. The submission deadline for the expression of interest (first stage) was 19 March 2013. Subsequently, the first-ranked applicant consortia from stage 1 have been invited to prepare and submit full project proposals to IMI JU by 26 July 2013.

eTOX: Integrating bioinformatics and chemoinformatics approaches for the development of expert systems allowing the *in silico* prediction of toxicities

eTOX is funded by the Innovative Medicines Initiative (IMI) Joint Undertaking, which started in 2010 and will run until December 2014. The consortium comprises 25 partners from academia and industry including SMEs. The aims of eTOX are to develop (i) a drug safety database from the pharmaceutical industry legacy toxicology reports and public toxicology data, and (ii) innovative *in silico* strategies and novel software tools to better predict the toxicological profiles of small molecules in the early stages of the drug development pipeline. This will be achieved by jointly storing and exploiting private data from the participating European Federation of

Pharmaceutical Industries and Associations (EFPIA) companies, as well as publicly available data, and by coordinating the efforts of specialists from EFPIA pharmaceutical companies, relevant SMEs and academic institutions. The proposed strategy includes a synergetic integration of innovative approaches in the following areas:

- database construction and management, including procedures and tools for protecting sensitive data;
- ontologies and text mining techniques, with the purpose of facilitating knowledge extraction from legacy preclinical reports and biomedical literature;
- chemistry- and structure-based approaches for the molecular description of the studied compounds, as well as their interactions with the anti-targets responsible for the secondary pharmacologies;
- prediction of DMPK features, since they are often related to the toxicological events;
- systems biology approaches in order to cope with the complex biological mechanisms that govern *in vivo* toxicological problems;
- computational genomics to afford the inter-species and inter-individual variability that complicates the interpretation of experimental and clinical outcomes;
- sophisticated statistical analysis tools required to derive the inevitably highly-multivariate QSAR models;
- development and validation (according to the OECD principles) of QSARs, integrative models, expert systems and meta-tools.

Project coordinator: Ferran Sanz, Fundació Institut Mar d' Investigacions Mèdiques, Spain

More information: http://www.etoxproject.eu/

Envisaged cooperation:

eTOX is operating in many fields that are related to the **SEURAT-1** Research Initiative. A representative of eTOX was invited to the **SEURAT-1** workshop 'Exploring Existing Databases for Modes-of-Action of Repeated Dose Systemic Toxicity' (see chapter 4.10.5.4). The databases and tools compiled and developed within eTOX may be an important resource for identifying key events within an adverse outcome pathway. Thus, database mining was identified as an important field for collaboration with eTOX and it was agreed that, once the **SEURAT-1** Research Initiative has completed the compilation of prototype mode-of-action descriptions, eTOX could provide some support in the refinement of mode-of-action descriptions through the elucidation of additional key events.

Important institutions that are active in **SEURAT-1**-related fields are compiled in the following descriptions:

EURL ECVAM: the European Union Reference Laboratory for Alternatives to Animal Testing

The European Commission's involvement in activities targeted toward the validation of alternative approaches to animal testing started in 1991, with the launch of ECVAM (the European Centre for the Validation of Alternative Methods), hosted by the Joint Research Centre, Institute for Health and Consumer Protection (IHCP). As of 2011, ECVAM's tasks were assigned to EURL ECVAM, and it is now part of the 'Systems Toxicology Unit' (STU) of the IHCP. Today, ECVAM provides the institutional basis to fulfil the requirements of the 'Directive 2010/63/EU on the protection of animals used for scientific purposes'. Following this, the aim of EURL ECVAM is twofold:

- to promote the scientific and regulatory acceptance of non-animal tests that are of importance to biomedical sciences, through research, test development and validation and the establishment of a specialised database service;
- to co-ordinate at the European level the independent evaluation of the relevance and reliability of tests for specific purposes, so that chemicals and products of various kinds (including medicines, vaccines, medical devices, cosmetics, household products and agricultural products) can be manufactured, transported and used more economically and more safely, while the current reliance on animal-based test procedures is progressively reduced.

EURL ECVAM collaborates with its closest partners in the field of validation through the 'International Collaboration on Alternative Test Methods' (ICATM). This agreement foresees intensified communication and collaboration during the planning and execution of validation studies on alternative methods, during peer review of these studies and with respect to the development of test method recommendations.

More information: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam

Existing collaboration:

The JRC (host institution of EURL ECVAM) is a key partner in several research projects (*SCR&Tox*, DETECTIVE, COSMOS) as well as in the coordination project COACH of the **SEURAT-1** Research Initiative. The experimental work in **SEURAT-1** aims to develop new test methods entering the pre-validation stage and, therefore, the involvement of ECVAM at an early stage is essential for the success of these activities. Furthermore, ECVAM may support the definition of cluster-level case studies, demonstrating that the new methods developed within **SEURAT-1** are fit for purpose.

CAAT-Europe: The Center for Alternatives to Animal Testing - Europe

The Center for Alternatives to Animal Testing - Europe (CAAT-Europe) was founded in 2009 as a transatlantic joint venture between the Johns Hopkins Bloomberg School of Public Health, Baltimore, USA, and the University of Konstanz. The University of Konstanz has 20 years of experience in the field of alternatives to animal testing. CAAT-Europe critically evaluates *in vivo*, *in vitro* and *in silico* approaches. The aim is to bring together the industrial and academic sectors that are involved in the development of toxicity tests in order to serve the needs for establishing alternative methods.

The objectives of CAAT-Europe are to (i) bring together industry and academics to address the needs for human-relevant methods; (ii) make use of strategic funds to fill gaps in the development and implementation of alternative methods; (iii) coordinate workshops and information days in Europe on relevant developments in the area of alternatives and toxicology; (iv) develop strategic projects with sponsors to promote human science and 'new toxicology'; (v) develop a joint education programme between the Johns Hopkins University in Baltimore and the University of Konstanz; (vi) set up transatlantic consortia for international research projects on alternative methods; and (vii) support *ALTEX* as the official journal of CAAT, the European Society for Alternatives to Animal Testing (EUSAAT), and the Transatlantic Think Tank for Toxicology (t4).

More information: http://cms.uni-konstanz.de/leist/caat-europe/

Existing collaboration:

Researchers from the **SEURAT-1** Research Initiative contributed as invited speakers to several workshops and symposia organised by the CAAT. The CAAT Europe Office and the **SEURAT-1** Office (COACH) are currently exchanging information about planned activities and, based on that, building a fruitful collaboration.

EBTC Europe: Evidence-Based Toxicology Collaboration

Following the effort in the US of creating an Evidence-based Toxicology Collaboration (EBTC) in 2011 (see below), a European counterpart to adapt Evidence-based Medicine (EBM) principles to toxicology has recently started. The kick-off meeting of EBTC Europe took place in conjunction with the EUROTOX Congress 2012.

More information: http://ebtox.com/eu-kickoff.html

OECD Chemicals Testing - Guidelines

The OECD (Organisation for Economic Co-Operation and Development) Guidelines for the Testing of Chemicals are a collection of the most relevant internationally agreed testing methods used for the safety assessment of chemicals. Different OECD working groups have

been established, addressing the various approaches in the field of toxicity testing, which will be briefly discussed below.

The (Quantitative) Structure-Activity Relationship [(Q)SAR] Project was launched in the early 1990s. This project has focused on the acceptance of (Q)SAR approaches for the evaluation of chemicals, focusing since 2004 particularly on the development of the OECD (Q)SAR Toolbox. This software was created for governmental instances and stakeholders in the chemical industry, in order to bridge the data gaps in (eco)toxicology. Version 2 of the Toolbox was released in 2010. It can be used for the identification of potential toxic mechanisms of chemicals, including their metabolites. The Toolbox comprises all regulatory endpoints and contains 'mechanistic profilers' for the identification of relevant mechanisms or modes-of-action.

The 'Molecular Screening for Characterisation Individual Chemicals and Chemical Categories Project' (Molecular Screening Project) was established in 2007 by the OECD in cooperation with the International Program on Chemical Safety (IPCS). The aim is to develop a strategy for prioritising of chemicals to be further tested, based on the molecular properties that are linked to potential toxicity. High-throughput screening (HTS) using *in vitro* assays and selected chemicals is being applied for the evaluation of specific pathways.

The emerging area of toxicogenomics is also being addressed by the OECD in collaboration with IPCS. The objectives are to (i) identify new biomarkers that are representative for specific pathways, and (ii) conduct surveys on existing toxicogenomic tools. The overall goal of these activities is the development of a strategy regarding the future application of toxicogenomics in the context of regulatory chemical safety assessment.

Finally, the OECD is very active in the field of adverse outcome pathway (AOP) developments, and has released some key documents outlining basic rules for establishing new AOPs as well as proposals for a common terminology (ontology) in this dynamic field.

More information: http://www.oecd.org/env/testguidelines

Existing collaboration:

Members from the **SEURAT-1** project COSMOS and the JRC are actively collaborating with the OECD in developing the AOP framework. The prototype AOPs developed and investigated within **SEURAT-1** may feed directly into the respective current OECD activities.

SCCS: Scientific Committee on Consumer Safety

The SCCS is organised in the European Commission's Directorate General for Health and Consumers. It provides opinions on health and safety risks of non-food consumer products

(such as cosmetic products and their ingredients) and services (such as artificial sun tanning). The SCCP releases the 'Notes of Guidance for the Testing of Cosmetic Ingredients and their Safety Evaluation', which is regularly updated according to scientific progress made.

More information: http://ec.europa.eu/health/scientific_committees/consumer_safety/

EFSA: European Food Safety Authority

As a consequence of a series of food crises, the European Food Safety Authority (EFSA) was set up in 2002 by the European Union as an independent agency for risk assessment and risk communication, covering all aspects associated with the food chain. EFSA aims to provide appropriate, consistent, accurate and timely communications on food safety issues to all stakeholders and the public at large, based on the Authority's risk assessments and scientific expertise. Nearly 460 people are currently engaged at EFSA, working in different food-related scientific fields, such as food and feed safety, nutrition, animal health and welfare, and plant protection. Giving independent scientific advice and assessing all risks concerning the food chain, EFSA plays a major role in Europe's food safety system.

More information: http://www.efsa.europa.eu/

5.2.2 International Activities

USA

Tox21: Toxicity 21

The 'Toxicology in the 21st Century' (Tox21) program is a joint initiative of the Environmental Protection Agency (EPA), the National Toxicology Program of the National Institute of Environmental Health Sciences (NIEHS), the National Institutes of Health (NIH), the National Center for Advancing Translational Sciences (NCATS) and the Food and Drug Administration (FDA), and is organised under the umbrella of the EPA's Computational Toxicology Research Program. Tox21 aims to develop high-throughput decision support tools for prioritising the thousands of chemicals that need toxicity testing. In this context, Tox21 develops, validates and translates innovative chemical testing methods that characterise toxicity pathways. The knowledge about toxicity pathways will then be used for prioritisation of chemicals that need to be further tested and the development of innovative *in silico* methods.

The general approach is to screen a large number of chemicals (approximately 10,000) using high-throughput screening assays at the NIH Center for Advancing Translational Science using innovative robotic technology. These data are then used to research, develop, validate and translate innovative chemical testing methods that characterise toxicity pathways. Ways to use new tools to identify chemically induced biological activity mechanisms are being

explored. This knowledge will then be used to prioritise the chemicals that need more extensive toxicological evaluation (i.e., the need for additional information). The experimental work is being accompanied by the development of models that can be used to more effectively predict how chemicals will affect biological responses. The different methods should be effectively combined as a toolbox of innovative chemical testing methods. Fifty or more ToxCast™ (see below) high-throughput screening assays in this enlarged chemical library should be conducted every year for the next several years. Finally, the challenge of being able to provide the data generated from the innovative chemical testing methods to risk assessors for making decisions about protecting human health and environment is addressed.

Four different working groups were established within Tox21: (i) Assays/Pathways Group, which is responsible for identifying key toxicity pathways/assays, incorporating of hepatic metabolism into *in vitro* assays, and establishing methods that account for interactions between compounds and pathways, as well as between cells (cell-to-cell interactions); (ii) Compounds Group, which is responsible for quality control issues and the establishment of two libraries, one containing the chemical structures of the 10,000 chemicals to be tested within Tox21, and another comprising water soluble compounds and mixtures to be tested in the future; (iii) Bioinformatics Group, which is responsible for interpreting data (response within and across assays and endpoints, respectively, and response pattern and relationships with adverse outcomes in *in vivo* tests) and ensuring the accessibility of data by the public; and (iv) Targeted Testing Group, which is responsible for evaluating the *in silico* methods and prioritisation schemes.

Scientific Coordinator: Robert Kavlock (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/Tox21/

Envisaged cooperation:

At the time of writing this report, **SEURAT-1** is organising a meeting with representatives from both Tox 21 and **SEURAT-1** (see section 5.3). The goal of this meeting is to discuss common interests as a basis for future collaboration.

ToxCast™: Screening Chemicals to Predict Toxicity Faster and Better

The US Environmental Protection Agency (EPA) launched ToxCast in 2007 as an important component of the EPA's Computational Toxicology Research Program for chemical screening. The aim is to develop a cost-effective approach for prioritising the vast number of chemicals that still need toxicity testing, and to predict the potential toxicity of chemicals. ToxCast uses advanced scientific tools to help understand how the processes of the human body are

impacted by exposure to chemicals and to determine which exposures are most likely to lead to adverse health effects. ToxCast is being developed in phases:

- Phase I (Proof of Concept) was completed in 2009 and it profiled roughly 300 well-studied chemicals (primarily pesticides) through the use of over 500 high-throughput screening assays. The chemicals screened in phase I already had extensive toxicity testing results from traditional chemical tests, mostly animal tests. Data from animal studies can be searched and queried using the EPA's Toxicity Reference Database (ToxRefDB, see below). Having both the ToxCast and animal testing results allows the EPA to compare results and determine if both screening processes make similar predictions.
- Phase II is currently screening 1,080 chemicals from a broad range of sources, including drugs, 'green' chemicals, chemicals in cosmetics and other consumer products.

Profiling through ToxCast means that a chemical is tested in over 500 existing high-throughput screening assays. The data are fed into the ToxCast database (ToxCastDB) and used for the elucidation of toxicity signatures. As ToxCast screens more chemicals, the EPA will be able to determine which combinations of high-throughput assays are best used as indicators for different types of potential toxicity that can lead to health effects such as chronic diseases.

Contact: David Dix (Deputy Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/toxcast/

Envisaged cooperation:

See entry above under Tox21.

ToxRefDB: Toxicity Reference Database

The Toxicity Reference Database (ToxRefDB) is another project that is organised under the umbrella of the EPA's Computational Toxicology Research Program. It was developed by the National Center for Computational Toxicology (NCCT) in collaboration with the EPA's Office of Pesticide Programs (OPP). The aim is to set up a comprehensive database of *in vivo* animal toxicity studies. This will allow for the establishment of links between toxicity pathways discovered in Tox21 and ToxCast (see above) and adverse outcomes *in vivo*.

The ToxRef database comprises several thousand animal toxicity studies, after testing hundreds of different chemical substances. ToxRefDB is the first database that makes chemical toxicity data accessible to the public, offering pesticide registration toxicity data and data from (sub)

chronic, cancer, reproductive and developmental studies. Furthermore, the database provides toxicity endpoints for the establishment of ToxCast predictive signatures.

More information: http://www.epa.gov/ncct/toxrefdb/

v-Liver™: The Virtual Liver Project

The Virtual Liver project was also established as a component of the EPA's Computational Toxicology Research Program. The aim is to estimate the potential of chemicals to cause chronic diseases such as cancer by means of a large-scale computer model simulating dynamic liver processes.

The mechanistic understanding of chemical effect networks will serve as the basis for modelling the key molecular, cellular and circulatory systems in the human liver. Health effects of chemicals over time will be estimated by means of a cell-based tissue simulator. Furthermore, the risk of human cancer through ingestion (the oral pathway) will be quantitatively estimated for selected chemicals (integration of physiologically based pharmacokinetic modelling (PBPK), cellular systems and molecular networks to simulate *in vivo* effects of chemicals), and 'virtual tissues' will be developed to evaluate the human health impact of chemicals using *in vitro* assays. Overall, the v-Liver project will predict chemically induced effects on the human liver on the level of virtual hepatic lobules using three interconnected systems: (i) simulation of micro-circulation and estimation of microdosimetry by using a vascular model network and *in vitro* data; (ii) simulation of key molecular events involved in determining phenotypic state of cells by means of *in vitro* data; (iii) simulation of the tissue response through a cellular systems model representing the complex interplay between hepatocytes and non-parenchymal cells.

More information: http://www.epa.gov/ncct/virtual_liver/

Other components of the EPA's Computational Toxicology Research Program

In addition to the above-mentioned projects that operate in the related fields of the **SEURAT-1** Research Initiative, the Computational Toxicology Research Program also comprises further components that will be just briefly mentioned here:

The EPA's online warehouse is called ACToR (Aggregated Computational Toxicology Resource). Comprising all publicly available chemical toxicity data, it can be used to find data on potential chemical risks to human health and the environment.

The ExpoCast™ project focuses on the environmental fate of chemicals to assess exposure routes. The project is closely related to ToxCast with the common goal of establishing a list of priority chemicals to be further tested and/or regulated.

The ToxPi project provides a platform to interconnect the information about toxicity pathways, dose estimates and chemical structures from other projects of the programme.

The v-Embryo project has its focus on developmental toxicity with the overall goal of developing prediction techniques for improved understanding of how environmental influences may impact unborn children. The project interacts with the ToxCast and the v-Liver projects.

Finally, the aim of the DSSTox (Distributed Structure-Searchable Toxicity) Database Network is to build a public data foundation for improved structure-activity and predictive toxicology capabilities.

More information: http://www.epa.gov/ncct/research_projects.html

DrugMatrix: A toxicogenomics and tissue library hosted by the National Toxicology Program

DrugMatrix is the scientific community's largest molecular toxicology reference database and informatics system. It is a current project of the National Institute of Environmental Health Sciences, one of the National Institutes of Health (NIEHS). DrugMatrix contains a graphic user interface for rapid scoring of genomic signatures of toxicity. DrugMatrix is populated with the comprehensive results of thousands of highly controlled and standardised toxicological experiments, in which rats or primary rat hepatocytes were systematically treated with therapeutic, industrial and environmental chemicals at both non-toxic and toxic doses and multiple exposure durations. The heart of the DrugMatrix database is large-scale gene expression data generated by extracting RNA from the toxicologically relevant organs and tissues and applying the RNA to the GE Codelink™ 10,000 gene rat array and, more recently, the Affymetrix whole genome 230 2.0 rat GeneChip® array. DrugMatrix contains toxicogenomic profiles for 638 different compounds.

DrugMatrix is publicly available. The primary value that DrugMatrix provides to the toxicology community is in its capacity to use toxicogenomic data to perform rapid toxicological evaluations. Further value is provided by DrugMatrix ontologies that help characterise mechanisms of pharmacological/toxicological action and identify potential human toxicities.

More information: https://ntp.niehs.nih.gov/drugmatrix/index.html

Envisaged cooperation:

A representative of the DrugMatrix project was invited to the **SEURAT-1** workshop 'Exploring Existing Databases for Modes-of-Action of Repeated Dose Systemic Toxicity' (see chapter 4.10.5.4). The DrugMatrix database tools may be an important resource for identifying key events within the selected **SEURAT-1** prototype adverse outcome pathways (AOPs).

Therefore, the DrugMatrix project could provide support in the refinement of these AOP descriptions through the elucidation of additional key events.

Tissue Chip for Drug Screening

To help streamline the therapeutic development pipeline, the National Center for Advancing Translational Sciences (NCATS) as part of the National Institutes of Health (NIH), in collaboration with the Defense Advanced Research Projects Agency and the US Food and Drug Administration, is leading an initiative to improve the process for predicting whether drugs will be safe in humans. The Tissue Chip for Drug Screening initiative aims to develop 3D human tissue chips that accurately model the structure and function of human organs, such as the lung, liver and heart. Once developed, researchers can use these models to predict whether a candidate drug, vaccine or biologic agent is safe or toxic in humans, and in a faster and more cost-effective way than current methods.

In July 2012, the NIH issued 17 awards, 10 of which will support studies to develop 3D cellular microsystems that represent a number of human organ systems. These bioengineered devices will be functionally relevant and will also accurately reflect the complexity of the tissue of origin, including genomic diversity, disease complexity and pharmacological response. The additional seven awards will explore the potential of stem and progenitor cells to differentiate into multiple cell types that represent the cellular architecture within organ systems. These could act as a source of cells to populate tissue chips.

More information: http://www.ncats.nih.gov/research/reengineering/tissue-chip/tissue-chip.html

NICEATM – ICCVAM: National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods - Interagency Coordinating Committee on the Validation of Alternative Methods

ICCVAM is an interagency committee of representatives from 15 US federal regulatory and research agencies that require, use, generate or disseminate toxicological and safety testing information. ICCVAM conducts technical evaluations of new, revised and alternative safety testing methods with regulatory applicability. ICCVAM also promotes the scientific validation and regulatory acceptance of safety testing methods that more accurately assess the safety and health hazards of chemicals and products and that reduce, refine (enhance animal well-being and lessen or avoid pain and distress) or replace animal use. NICEATM administers ICCVAM and provides scientific and operational support for ICCVAM-related activities. NICEATM also conducts independent validation studies to assess the usefulness and limitations of new, revised and alternative test methods and strategies.

ICCVAM has contributed to the approval or endorsement of 43 alternative safety testing methods by federal regulatory agencies and international organisations since its establishment in 1997. ICCVAM has also identified critical research, development and validation efforts needed to further advance numerous other alternative methods.

In 2008 NICEATM and ICCVAM published a five-year plan for the years of 2008 through to 2012. The current plan addresses (i) the identification of areas of high priority for new and revised non-animal and alternative assays to reduce, refine (enhance animal well-being and lessen or avoid pain and distress) and replace the use of animals in testing, and (ii) the research, development, translation and validation of new and revised non-animal and other alternative assays for integration into federal agency testing programs. NICEATM and the National Institute of Environmental Health Sciences (NIEHS) and the invited public can provide comments that can be considered by the ICCVAM and agencies' program offices in updating this five-year plan. A request for comments was published in the Federal Register on 21 November 2011 (76 FR 71977). Comments were requested by 15 January 2012.

More information: http://iccvam.niehs.nih.gov/

PSTC: Predictive Safety Testing Consortium (InnoMed)

The PSTC is a public–private partnership supervised by the Critical Path Institute (C-Path) as an independent, non-profit institute, which was created by the University of Arizona and the US Food and Drug Administration (FDA) in 2005. PSTC provides a platform for pharmaceutical companies to share and validate each other's safety testing methods under advisement of the US Food and Drug Administration (FDA) and its European counterpart, the European Medicines Agency (EMA). PSTC is creating new tools for safety assessment in drug development useful for pharmaceutical scientists, regulators and clinicians. Currently, the PSTC has 17 corporate members.

The PSTC has two main objectives: (i) the identification and cross-qualification of new and improved pre-clinical safety testing methods through a collaboration of scientists from the pharmaceutical industry, academia and regulators (FDA, EMA, Japanese Pharmaceuticals and Medical Devices Agency); and (ii) the facilitation of the development of new regulatory processes for approving such testing methods. The PSTC is subdivided into six working groups addressing the following areas of safety: kidney injury, liver injury, skeletal muscle injury, vascular injury, cardiac hypertrophy, and carcinogenicity.

Director: Elizabeth Walker (Critical Path Institute, Tucson, USA)

More information: http://www.c-path.org/pstc.cfm

HESI: Health and Environmental Sciences Institute

The Health and Environmental Sciences Institute (HESI) is a non-profit, scientific organisation located in Washington D.C., USA. HESI was established in 1989 as a global branch of the International Life Sciences Institute (ILSI). HESI's intention is to bring together different research groups from industry, government and academia to advance the understanding of scientific issues in the field of human health, toxicology, risk assessment and the environment. HESI developed a programme for laboratory studies, workshops and conferences that is currently used by 43 companies representing different branches of the chemical industry. This programme is co-financed through grants from the private sector as well as from various international regulatory institutions.

Executive Director: Michael P. Holsapple (Health and Environmental Science Institute, Washington D.C., USA)

More information: http://www.hesiglobal.org/

CAAT: Centre for Alternatives to Animal Testing

The Centre for Alternatives to Animal Testing (CAAT) is located within the Johns Hopkins Bloomberg School of Public Health in Baltimore. It was established in 1981 through a grant from the Cosmetic, Toiletry, and Fragrance Association (CTFA) (now the Personal Care Products Council). Similarly to the European counterpart described above, CAAT's vision is to be a leading force in the development and use of methods following the 3Rs principle (reduction, refinement and replacement) in all involved sectors (research, testing and education). Consequently, CAAT supports research for the development and validation of new *in vitro* test methods and other alternatives, organises discussion to enhance acceptance of such new methods, distributes information to academia, government, industry and the general public (for instance through the *ALTEX* journal), and organises training courses in the application of innovative methods in toxicity testing.

More information: http://www.caat.jhsph.edu

EBTC: Evidence-Based Toxicology Collaboration

The Evidence-Based Toxicology Collaboration has taken up the challenge of translating evidence-based approaches from medicine to toxicology. The Collaboration has closely coordinated steering committees in the US and Europe with members drawn from government agencies, academia and industry. The EBTC will further the conceptual development of evidence-based toxicology, set priorities, raise awareness and create working groups. Three working groups are currently active: (i) the Zebrafish Work Group (formed in late 2012 to

carry out a systematic review of the Zebrafish Embryo Test as a predictor of developmental toxicity); (ii) the Methods Work Group (to identify and adapt methods from evidence-based medicine and health care that are applicable to evidence-based toxicology, as well as develop new methods as necessary); (iii) the Governance and Work Processes Work Group (to identify, recommend and implement appropriate administrative structures and procedures to facilitate the activities of the EBTC). The working groups produce guidance documents – tailored to toxicology – on conducting systematic reviews and their components, including data appraisal and data synthesis, as well as on the application of evidence-based tools to various toxicological practices, such as assessing the hazards and risks of exposure to individual chemicals and evaluating the performance of toxicological test methods. The EBTC will also undertake case studies to illustrate how evidence-based approaches can address these topics. The EBTC will evolve into an umbrella organisation facilitating the application of evidence-based approaches to toxicology.

The Center for Alternatives to Animal Testing (CAAT; see above), located at the Johns Hopkins University in Baltimore, US, has served as the EBTC's secretariat since the latter's founding in 2011.

More information: http://www.ebtox.com/

JAPAN

JaCVAM: Japanese Center for the Validation of Alternative Methods

JaCVAM is part of the Office for New Testing Method Assessment in the Division of Pharmacology of the Japanese National Biological Safety Research Centre (NBSRC) and the National Institute of Health Sciences (NIHS). JaCVAM is responsible for the evaluation of innovative testing methods following the 3Rs principle in the field of chemical toxicity screening and thereby for chemical safety assessment in Japan. JaCVAM's agenda also comprises the establishment of guidelines for alternative testing methods, with special emphasis on international collaborations for the development of harmonised experimental protocols (e.g., correlation with OECD guidelines). For that, JaCVAM organises international workshops and disseminates the respective information regarding alternative testing methods. Furthermore, representatives of the US National Toxicology Program, Health Canada, Japan (JaCVAM) and the EU (ECVAM) signed a memorandum of cooperation in 2009 with the aim of establishing an International Cooperation on Alternative Test Methods (ICATM). This was done in order

"to expand and strengthen cooperation, collaboration and communication among national validation organisations on the scientific validation and evaluation of new alternative testing methods proposed for regulatory health and safety assessments" (Memorandum of Cooperation, http://jacvam.jp/en_effort/en_icatm.html).

More information: http://jacvam.jp

TG-GATEs: Genomics Assisted Toxicity Evaluation System

TG-GATEs is a project of the Laboratory of Toxicogenomics Informatics hosted by the Japanese National Institute of Biomedical Innovation. The first five-year collaborative studies in the Toxicogenomics Project by the government and pharmaceutical companies started in 2002, in which rats were exposed to chemicals (mainly medicines) and gene expression in the liver (kidney in some cases) was measured by Affimetrix's GeneChip and collected together with classical toxicological data. Experiments were also done with rat and human hepatocytes and more than eight hundred million gene expressions for more than 150 chemicals were obtained by 2007. The data were combined with analysis and prediction systems established under the name of TG-GATEs (Genomics Assisted Toxicity Evaluation system). In order to utilise this system effectively, the second stage of the Toxicogenomics Informatics Project was started in 2007.

Data collected by TG-GATEs is publicly available (http://toxico.nibio.go.jp/open-tggates/search.html).

More information: http://www.nibio.go.jp/english/part/fundamental/detail13.html

5.2.3 Meetings and Symposia

FOCUS ON ALTERNATIVE TESTING

European Partnership for Alternative Approaches to Animal Testing

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a joint initiative from the European Commission, European trade associations and individual companies. The EPAA organises annual conferences and workshops, which are announced on its webpage (see below). Most important for the **SEURAT-1** Research Initiative in the previous year were the following activities:

- A workshop focusing on acute toxicity on 28 September 2012 in Brussels, Belgium: 'How could classification and labelling decisions in the agrochemical and chemical sectors be made if stand-alone acute toxicity testing were prohibited?';
- The EPAA 2012 Annual Conference, entitled 'Development and Implementation of 3R methodologies through International Cooperation', which took place in Brussels, Belgium, on 16 November 2012. At this conference, a 'Memorandum of Understanding' between the EPAA and the US-based non-profit Institute of *In Vitro* Studies was signed, establishing a strategic partnership dedicated to the international dissemination of alternative techniques for safety evaluation;

➠ The EPAA Stem Cells Forum organised an event entitled 'New Perspectives on Safety Project – Stem Cells', which was held on 25 April 2012 in London, UK.

Full workshop reports are available on the EPAA homepage. The EPAA 2013 Annual Conference will be held on 13 November in Brussels.

More information: http://www.epaa.eu.com

ecopa

Similarly, the European Consensus-Platform for Alternatives (ecopa) has been established to stimulate research into alternatives to animal experimentation and enforce the acceptance of alternatives in experimental practice. The ambition is to act as a pan-European platform, integrating people from different sectors, such as animal welfare, industry, academia and governmental institutions. As one of its main activities, ecopa supports the organisation of workshops in the field.

More information: http://www.ecopa.eu/

Scientific Roadmap for the Future of Animal-free Systemic Toxicity Testing Workshop

Host: US FDA

Date: 30–31 May 2013

Location: College Park, Maryland, USA

The scientific roadmap was the product of a workshop that took place in October 2011, held under the auspices of the Transatlantic Think Tank for Toxicology (t4). Five whitepapers on systemic toxicity were prepared for workshop, taking a broad perspective on how to make further progress. The outcome was published as 'A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing', by Basketter et al. in January 2012 issue of *ALTEX*. The roadmap was favourably reviewed and discussed at a follow-up open meeting in Brussels in March 2012, co-organised by numerous organisations and attended by some 150 experts.

The May 30–31 workshop provided a similar forum but in a US setting. The program included ample opportunity for public discussion of the roadmap, as well as its possible updating in light of more recent developments.

More information: http://www.fda.gov/ScienceResearch/AboutScienceResearchatFDA/ucm351144.htm

Mondial Research Group meeting on Reduced Animal Testing

Date: 25–26 July 2013

Location: Zurich, Switzerland

Although most the experiments performed on animals are regarded as important for furthering of human and veterinary science, there is a strong movement from within the scientific community to develop methods that do not rely on animals. However, it still may take a long time before all animal experiments can be replaced. The meeting focused, therefore, on options to reduce both the number and suffering of experimental animals.

Main themes for discussion were:

- An in-depth Study of the 3Rs;
- Relative and Absolute Replacement Models;
- Difficulties of Extrapolating Results to the Human Situation;
- *In vitro* Methods: Replacement or Addition to Animal Testing;
- Computer Modelling, Biochemical Techniques and *in vitro* Methods;
- The Refinement and Reduction of Suffering of Experimental Animals before, during and after an Experiment.

More information: www.mondialresearchgroup.com/

18th European Congress on Alternatives to Animal Testing in Conjunction with the 15th Annual Congress of EUSAAT

Date: 15–18 September 2013

Location: Linz, Austria

The goals of the European Society for Alternatives to Animal Testing (EUSAAT) are to support: (i) the dissemination and validation of alternative methods to animal testing; (ii) the promotion of research in the field of the 3Rs (refine, reduce, replace); (iii) the reduction of the use of animals for tests in the field of education and continuing education; (iv) the reduction of sufferance and stress of laboratory animals by better breeding, keeping, test planning and other accompanying measures; (v) the provision of specialist guidance and expert opinions for public and private organisations, companies and universities; (vi) suitable information of the public and the media.

Main themes of the congress for discussion are:

- ESNATS – Embryonic Stem Cells;
- Progress in 3Rs Research – EU FP6 and FP7 Projects;

- Directive 2010/63/EU and Transposition into National Legislation;
- Basic and Biomedical Research;
- Regulatory Toxicity Testing (REACH, 2013 Marketing Ban for Cosmetics);
- Specific Toxicological Endpoints: Local Toxicity (Eye and Skin), Repeated-dose Toxicity, Inhalation Toxicity, Sensitisation and Immunotoxicity, Reproductive and Developmental Toxicity and Endocrine Disrupters, Genotoxicity and Carcenogenicity;
- Nanotoxicology;
- Ecotoxicology (Endocrine Disruptors);
- Toxicology in the 21st Century;
- Human-on-a-Chip;
- Disease Models;
- 3Rs go 3D;
- Drugs, Vaccines and Medical Devices;
- Ethical and Legal Issues.

More information: http://www.eusaat.org/

INVITROM: *In Vitro* Models: The Cell is the Limit?

Date: 16 October 2013

Location: Breda, The Netherlands

INVITROM is the International Society for *In Vitro* Methods. Their mission is the promotion of the development, application and acceptance of *in vitro* models in biomedical research. The ambition of INVITROM is to accelerate the development of models, paradigms and strategies through information exchange. Moreover, INVITROM wants to promote the development, acceptance and implementation of these methods amongst others, by stimulating the collaboration between the research institutes and industry and by informing the regulatory bodies. In order to achieve these goals, INVITROM uses several communication tools including workshops and symposia.

The INVITROM Annual Symposium 2013 will cover a variety of aspects including the fields of PBPK modelling and neurotoxicity.

More information: http://www.invitrom.org/symposium2.html

IVTS: *In Vitro* Toxicology Society

Date: 4–5 November 2013

Location: Leicester, England

The IVTS was founded under a constitution in 1988 for scientists active in the study, practice or development of *in vitro* toxicology. The aims of the IVTS are (i) to encourage alternative approaches to animal testing and promoting the 3Rs; (ii) to provide a forum for discussion for scientists actively involved in the study, practice or development of *in vitro* toxicology; (iii) to arrange scientific meetings on the subject of *in vitro* toxicology and its practical applications; (iv) to promote an exacting scientific approach to the practice of *in vitro* toxicology; and (v) to encourage participation of new student scientists in the field of *in vitro* toxicology.

The IVTS organises their annual meeting in November, with topics ranging from regulatory issues through to innovative *in vitro* models and provides an excellent forum for sharing information and cross-sector and cross-disciplinary collaboration. The main session topics for the IVTS 2013 meeting are:

- High Content Biology;
- Repeated Dose Toxicology (this session will be fully covered by **SEURAT-1**);
- Ecotoxicology;
- Tissue Modelling.

More information: http://www.ivts.org.uk/site/

IVTIP: *In Vitro* Testing Industrial Platform

Date: 7–8 November 2013

Location: Monaco

The *In Vitro* Testing Industrial Platform was established in December 1993 with the aim of (i) advising European bodies (such as the European Commission and European Parliament) on industrial requirements for current and future research projects in *in vitro* testing; (ii) optimising the industrial value of EU-funded research projects; and (iii) encouraging the further development, validation and regulatory acceptance of tests based on this research. IVTIP members represent companies in the following sectors: chemicals, cosmetics, consumer products and pharmaceuticals. IVTIP organises two plenary meetings each year, where its members meet with invited regulators, SMEs and academics active in the field of *in vitro* testing. During the meetings state-of-the-art presentations are given and position papers are drafted.

More information: http://www.ivtip.org/

ESTIV2014: International Conference of the European Society of Toxicology *In Vitro*

Date: 10–13 June 2014

Location: Egmond aan Zee, The Netherlands

The European Society of Toxicology *In Vitro* is Europe's leading organisation working to strengthen the scientific network of *in vitro* toxicologists and promotes *in vitro* toxicology, both scientifically and educationally, in all countries of Europe. ESTIV2014 is jointly organised with the International Society of *In Vitro* Methods (INVITROM). ESTIV and INVITROM aim to create a forum for scientists to discuss and exchange knowledge in a uniquely friendly atmosphere. The objective of the congress is also to promote interaction between junior and senior scientists, students and toxicologists from European companies, government and universities involved in the development and use of *in vitro* methods in toxicology and toxicity testing.

The ESTIV2014 congress will have an attractive scientific programme focused on the slogan: 'Making sense of *in vitro* methods'. Emphasis will be specifically on how new technologies can strengthen the interpretation and application of *in vitro* methods in toxicological research and risk assessment. Session themes are:

- Advanced *in vitro* Models;
- Body-on-a-Chip;
- Stem Cell Research;
- The New Paradigm in Toxicological Risk Assessment;
- New Molecular Mechanisms and Biotechnologies in Toxicology;
- Nanotoxicology.

It is planned that this conference will be held in conjunction with the second **SEURAT-1** Summer School. Furthermore, NOTOX is organising its first satellite meeting in conjunction with ESTIV on 10 July 2014. The NOTOX Satellite Meeting will bring together an interdisciplinary panel of scientists to present current efforts, challenges and future directions for long-term repeated dose toxicity assessment using *in vitro* organotypic hepatic cultures. The meeting programme will focus on systems biology approaches in predicitve toxicology using computer modles.

More information: http://www.estiv2014.org/

9th World Congress on Alternatives and Animal Use in Life Sciences

Date: 24–28 August 2014

Location: Prague, Czech Republic

The Congress provides a forum supporting the ethical use of animals in chemical testing, as well as scientific exchange regarding the development of innovative experimental methods. The motto of the 9th World Congress is: 'Human Science in the 21st Century'.

Main themes for discussion are:

- New Technologies;
- Predictive Toxicology and Safety Assessment (including a dedicated **SEURAT-1** session);
- 3Rs in Academia and Education;
- Communication, Dissemination and Data Sharing;
- Efficacy and Safety Testing of Drugs and Biological;
- Human Relevance (*in vivo, in vitro*);
- Ethics;
- Refinement and Welfare;
- Global Cooperation, Regulatory Acceptance, Standardisation.

More information: http://www.wc9prague.org/

OTHERS IN THE FIELD

12th Annual World Pharma Congress (WPC)

Organisers: Cambridge Healthtech Institute

Date: 4–6 June 2013

Location: Philadelphia, USA

After a dozen years, covering various topics for the pharmaceutical industry, the World Pharma Congress has found its niche in effectively covering the latest preclinical strategies and technologies for driving better predictions. Building on its experience covering drug targets, high-throughput screening, lead optimisation, ADME and drug safety, the event is now primed to move further downstream into early formulations and drug delivery.

The Congress motto 2013 was: 'Advancing Preclinical Predictions, Faster Clinical Decisions'. It brought together a mix of scientists and clinicians from academia and industry to facilitate active brainstorming and networking on challenging issues in the field. The conference also included contributions from leading technology and service providers on the latest tools and services available in the marketplace.

Main themes for discussion were:

- Predictive Preclinical Models in Oncology;
- Targeting Pain with Novel Therapeutics;
- Formulation and Drug Delivery;
- Integrated Drug Safety Risk Assessments;
- Molecular Imaging in Drug Discovery and Development.

More information: http://www.worldpharmacongress.com/

13th International Congress of Toxicology in Conjunction with the 2nd International Conference on Environmental OMICS

Date: 30 June–4 July 2013

Location: Seoul, South Korea

The theme for the conference was 'From Basic Science to Clinical and Environmental Outcomes'. This conference encompassed novel approaches and technologies being used for properly assessing the safety of and toxicity and risks to human health. The scientific programme consisted of 28 symposia (one dedicated to 'The Evidence-Based Toxicology Collaboration'), 8 workshops, 2 round-table discussions (one entitled 'Towards Improved Chemical Risk Assessment: International Challenges and Opportunities') and related debate and poster sessions.

More information: http://www.ict2013seoul.org/

49th Congress of the European Societies of Toxicology

Date: 1–4 September 2013

Location: Interlaken, Switzerland

The Federation of European Toxicologists & European Societies of Toxicology (EUROTOX), with about 7,000 members of different countries, was founded in 1985. EUROTOX organises an annual congress presenting topics covering the latest scientific and regulatory developments with the aim of encouraging future work in toxicology (scientifically as well as educationally).

Main themes for discussion include:

- 3D Cell Models in Drug Safety: With Better Tool to Better Drugs?;
- Alternative Test Methods: Challenges and Regulatory Application;

- Profiling the Toxicity of New Drugs: A non Animal-based Approach Integrating Toxicodynamics and Biokinetics;
- Early Safety Assessment: Considerations and Strategies in Drug Discovery;
- Renal Toxicology – Epidemiology, Mechanisms and Risk Assessment;
- Stem Cell Derived Tissues in Safety Assessment;
- RISK21: Novel Thinking for 21st Century Risk Assessment;
- Cardiovascular Toxicity in Drug Discovery and Development;
- New Developments in '-omics' for Use in Risk Assessment;

More information: http://www.eurotox2013.com/

53rd Annual Meeting of the Society of Toxicology (SOT)

Date: 23–27 March 2014

Location: Phoenix, USA

The SOT Annual Meeting is the most comprehensive forum for highlighting premier scientific presentations that span the discipline of toxicology. From the essential knowledge to the latest advances, the scientific sessions, including platform sessions, poster presentations, and plenary talks, provide access to the important information of the field.

Main themes for discussion are:

- Advancing Clinical and Translational Toxicology and Application of Biomarkers;
- Enhancing Strategies for Risk Assessment;
- New Science and Perspectives Surrounding Environmental and Occupational Exposures;
- Safety Assessment: Mechanisms and Novel Methods;
- Stem Cell Models for Integrated Biology.

More information: http://www.toxicology.org/AI/MEET/AM2014/

5.3 SEURAT-1 Meets Tox21

The COACH Team

5.3.1 Workshop Objectives

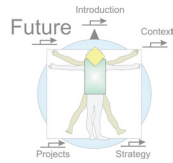
The workshop '**SEURAT-1** meets Tox21' with a limited number of invited participants from both projects, took place at the European Commissions Joint Research Centre (JRC) site in in Ispra, Italy, on 25–27 June 2013.

The objective of the workshop was to facilitate a practical exchange of scientific information between the **SEURAT-1** Research Initiative and the Tox21 consortia, including ToxCast, with the intention of stimulating cooperation between partner organisations, either on a multilateral or bilateral basis. The agenda was based on topics of common interest and aimed to divide the time between targeted presentations and related discussions. It is foreseen that eventual cooperation could take place on a more technical level, for example through the sharing of research materials (data, cells, assays, models, etc.), or on a application level, for example through the linking up of **SEURAT-1** case studies with Tox21 projects. The outcome of the workshop was a concise set of mutually agreed recommendations for cooperation activities and a list of proposed follow-up actions.

As described above (see section 5.2.2), the 'Toxicology in the 21st Century' (Tox21) program is a US federal collaboration involving the Environmental Protection Agency (EPA), National Center for Advancing Translational Sciences (NCATS), National Institute of Environmental Health Sciences (NIEHS), the National Institutes of Health (NIH), and Food and Drug Administration (FDA). The goal of Tox21 is to quickly and efficiently test whether certain chemical compounds have the potential to disrupt processes in the human body that may lead to adverse health effects, using *in vitro* high-throughput screening and computational methods.

ToxCast™ uses advanced scientific tools to help understand how human body processes are impacted by exposure to chemicals and to determine which exposures are most likely to lead to adverse health effects. The US Environmental Protection Agency (EPA) launched ToxCast™ in 2007 to develop ways to predict potential toxicity of chemicals and to develop a cost-effective approach for prioritising the thousands of chemicals that need toxicity testing.

The **SEURAT-1** idea of building integrated testing strategies for toxicity prediction based on alternative methods is of course closely related to Tox21 activities, and it is of strong mutual interest to start discussing possible collaborations between partners of the two initiatives.



5.3.2 Agenda

Day 1

International efforts to advance safety assessment science using animal-free methods: The purpose of the session was to introduce the ToxCast, Tox21 and **SEURAT-1** research programmes in terms of their strategic aims, structure and research focus, and to touch on selected highlights. Time was also dedicated to looking towards Horizon 2020 and exploring the motivation and possibilities for cooperation between the USA and EU in the area of health related research.

14:00-14:10	Welcome (Krzysztof Maruszewski; Director IHCP)
14:10-14:30	The ToxCast programme of the US EPA (Richard Judson; EPA)
14:30-14:50	Toxicity testing in the 21st century – an introduction to Tox21 (Ray Tice; NIEHS)
14:50-15:10	Non-animal methods for chronic systemic toxicity assessment – an introduction to **SEURAT-1** (Michael Schwarz; COACH)
15:10-15:30	Prospects for international cooperation in health related research under Horizon 2020 (Bernard Mulligan; European Commission, DG RTD)
15:30-16:00	*Coffee*

Chemical inventories: This session focused on describing the chemical inventories assembled for ToxCast, Tox21 and **SEURAT-1** and the different approaches employed for chemical selection.

16:00-16:30	The ToxCast and Tox21 chemical libraries (Matt Martin; EPA)
16:30-17:00	Selection of the **SEURAT-1** gold compounds (Barry Hardy; ToxBank)
17:00-17:30	The COSMOS cosmetics inventory (Mark Cronin; COSMOS)
17:30-18:00	Discussion

Day 2

***In vitro* assays and test systems:** This session provided a detailed overview of the various *in vitro* assays and test systems that are being developed and applied within the consortia, including the cell/tissue models, biomarkers and measurement techniques that they are based on. Attention was given to the rationale used to select the assays and the mechanistic information derived from them.

09:00-09:30	The biological space covered by the ToxCast and Tox21 assays (Matt Martin; EPA)
09:30-10:00	The ultra-HTS workflow at NIH-NCATS (Anton Simeonov; NCATS)
10:00-10:30	Discussion
10:30-11:00	*Coffee*
11:00-11:30	Screening for mitochondrial toxicants using ultra-HTS (Ruili Huang; NCATS)
11:30-12:00	Metabolically functional, polarised models of proliferating human hepatocytes: Round 1 of Gold Compound Screen (Yakoov Nahmias; *HeMiBio*)
12:00-12:30	Stem cell-based assays for toxicity testing (Susanne Bremer; *SCR&Tox*)
12:30-13:30	*Buffet lunch*
13:30-14:00	2D- and 3D-based assays that support systems biology modelling (Elmar Heinzle; NOTOX)
14:00-14:30	Biomarker discovery for novel *in vitro* assays (Dimitry Spitkovsky; DETECTIVE)
14:30-15:00	Strategies for mining mechanistic information from transcriptomics datasets (Scott Auerbach; NIEHS)
15:00-15:30	Discussion

Computational tools and predictive toxicity case studies: This session reviewed the various computational approaches being employed to predict toxicokinetic and toxicodynamic properties of chemicals and also to integrate property data from *in vitro* and computational methods in order to make toxicity predictions.

16:00-16:40	Predictive toxicity case studies based on ToxCast data (Richard Judson; EPA)
16:40-17:20	Computational tools being employed within **SEURAT-1** (Mark Cronin; COSMOS)
17:20-17:30	Candidate predictive toxicity case studies being considered for **SEURAT-1** (Maurice Whelan; COACH)
17:30-18:00	Discussion

Day 3

Safety assessment: This session focused on how the output and efforts of this research can be best exploited for the purposes of chemical safety assessment. The intention was to explore how data from 'non-standard' (non-animal) methods can be used in different decision-making contexts and frameworks.

09:00-09:30	How ToxCast and Tox21 data can inform the chemical risk assessment process (Richard Judson; EPA)
09:30-10:00	Safety assessment related goals of **SEURAT-1** including case studies (Andrew White; **SEURAT-1** Safety Assessment Working Group)
10:00-10:30	Discussion
10:30-11:00	*Coffee*
11:00-12:00	Recommendations and Actions (including practicalities such as ways to interact and share information/data)
12:00-12:15	Wrap-up

5.3.3 Participants

Hans Jürgen Ahr *(Bayer AG; SEP **SEURAT-1**)*

Scott Auerbach (*National Institute of Environmental Health Sciences, USA*)

Emilio Benfenati (*Istituto di Ricerche Farmacologiche Mario Negri; ToxBank*)

Elisabet Berggren (*Joint Research Centre; COACH*)

Susanne Bremer (*Joint Research Centre; SCR&Tox; DETECTIVE*)

Warren Casey (*NTP Interagency Center for the Evaluation of Alternative Toxicological Methods, National Institute of Environmental Health Sciences, USA)*

Ian Cotgreave *(AstraZeneca; SEP **SEURAT-1**)*

Mark Cronin *(Liverpool John Moores University; COSMOS)*

Christian Desaintes (*European Commission, DG Research & Innovation*)

David Dix (*Environmental Protection Agency, USA*)

Barry Hardy (*Douglas Connect; ToxBank*)

Elmar Heinzle *(Saarland University, Germany; NOTOX)*

Ruili Huang (*National Institutes of Health, National Center for Advancing Translational Sciences, USA*)

Richard Judson (*Environmental Protection Agency, USA*)

Derek Knight (*European Chemicals Agency; SEP* ***SEURAT-1***)

Catherine Mahony (*Procter & Gamble; SEP* ***SEURAT-1***)

Matthew Martin (*Environmental Protection Agency, USA*)

Bernard Mulligan (*European Commission, DG Research & Innovation*)

Yaakov Nahmias (*The Hebrew University of Jerusalem; HeMiBio*)

Jens Niklas (*Insilico Biotechnology AG; NOTOX*)

Alexandre Pery (*French National Institute for Industrial Environment and Risk; COSMOS*)

Michael Schwarz *(University of Tuebingen, Germany; COACH)*

Anton Simeonov (*National Institutes of Health, USA*)

Dimitry Spitkovsky (*University of Cologne, Germany; DETECTIVE*)

Daniella Steel (*Cellectis AB; SCR&Tox*)

Russell Thomas (*The Hamner Institutes for Health Sciences, USA; SEP* ***SEURAT-1***)

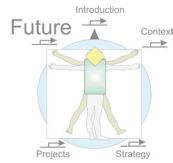
Raymond Tice (*National Institute of Environmental Health Sciences, USA*)

Leo van Grunsven (*Vrije Universiteit Brussel, Belgium; HeMiBio*)

Mathieu Vinken (*Vrije Universiteit Brussel, Belgium; HeMiBio; DETECTIVE*)

Maurice Whelan (*Joint Research Centre; COACH*)

Andrew White (*SEAC, Unilever PLC*)



5.4 Beyond SEURAT-1: Approach Based on Understanding Interaction of Chemicals at Fundamental Biological Levels

George P. Daston, Russell S. Thomas, Michael Schwarz

5.4.1 Setting the Stage

The replacement of repeated-dose testing with alternative models is a daunting task. Repeated-dose studies are intended to be comprehensive evaluations of the potential for a chemical to affect physiological function in virtually every organ/tissue in the body. It is typically the basis for establishing safe levels of exposure for a chemical that may be encountered every day for a lifetime. Up to now the tests have been conducted in laboratory animal models in which the readout has largely been histopathology and clinical chemistry. The value of these studies is that the readouts – histopathology of organs and/or abnormal clinical chemistry parameters – are believed to be analogous to human disease states. The downside of these studies is that they generally do not provide any information about the steps that occur between interaction of the chemical with its initial target and the ultimate outcome, thereby often raising questions about the relevance of the animal's response for human safety and risk assessment. Besides ethical objections, animal studies are also costly and time-consuming; therefore, relatively few chemicals have been fully tested for toxicity compared with the number that is believed to be in the environment.

Most current non-animal based ('alternative') methods attempt to 'mimic' specific *in vivo* organ or tissue responses to a test chemical in a one-to-one replacement strategy. Future toxicology, aiming at a better science and an associated reduction in the use of experimental animals, will require a complete shift in paradigm. This shift in paradigm consists of a new definition of 'adversity', which will no longer be defined as an apical manifestation of an effect. Rather, reductionist methods will evaluate the initial interaction of chemical with some aspect of the living system, and the responses that follow at the molecular and cellular levels. If so, future testing will aim to identify the molecular targets of test chemicals and the quantitative determination of the degree of perturbation of cell signalling or other cellular networks caused by interaction of the chemical with its target/s. The potential effect on health/disease is inferred, based on the known connections between molecular event and ultimate outcome. Such a definition of adversity at the molecular level opens a new understanding of toxic effects. Of course, in order for this approach to be ultimately successful, it is necessary (i) to have enough reductionist assays to cover the range of targets/modes-of-action present in humans; (ii) to have sufficient understanding of the relevance of the mode-of-action to outcome to be able to

infer this outcome; and (iii) to be able to extrapolate from concentrations that elicit a response *in vitro* to comparable dose levels *in vivo*.

In order to address the first requirement, it is necessary to know the number of targets or modes of toxicity. The number is finite, although not known at this point. It may be possible to take clues from pharmacology, where scientists have identified a 'druggable genome', the sum of all targets that have the capability of interacting with small molecules. In addition, large ligand-target, hereditary disease and gene expression databases can be mined to piece together the potential biological targets for toxicants. Many of the initial targets may converge into a reduced number of 'critical' downstream signalling cascades, which would then be the focus of an *in vitro* test system.

Toxicity, at a chemical level, can be broken down into three categories: (i) strong reactivity leading to covalent binding or oxidation of the target; (ii) strong interactions that are relatively selective and qualitatively similar to the actions of drugs; and (iii) weak interactions that are relatively non-selective and can perturb multiple cellular targets within a narrow concentration range. Correctly binning the chemicals into each of the three categories will serve as the first step for evaluating the potential mode-of-action of a chemical and direct which downstream batteries of high-throughput assays or high-content (e.g., gene expression) assays may be appropriate.

The second requirement is to be able to 'connect the dots' between effects at a molecular/cellular level and ultimate outcome. This is the task of constructing adverse outcome pathways that will be built from mode-of-action information along with reviews of the literature and systems biology approaches, coupled with cell and tissue culture.

The third requirement, that test concentrations be relevant to human exposure levels, and are able to be extrapolated from *in vitro* concentration to *in vivo* dosage, is being addressed through the development of screening-level comparisons (*Wetmore et al., 2012*) or reverse dosimetry methods (*Yoon et al., 2012*).

The vision, then, for an alternative to repeated-dose toxicity testing is a series of assay systems that concentrate on maximal coverage of the mode-of-action, coupled with higher-order models when needed either to elucidate potential higher-order outcomes or to investigate interactions between modes-of-action that could lead to quantitatively different outcomes than either mode alone. The data stream may come from batteries of high-throughput tests, high-information-content procedures such as transcriptomics, or even from SAR and QSAR models, as long as they are built on sound principles. These data would then be analysed with a decision matrix that will determine whether a tested chemical has any specific toxicity, and if so, what the likely outcomes could be. In some cases the adverse outcome pathways will be sufficiently established, or there will be sufficient supporting information from close analogues of the chemical that have already been tested *in vivo*, to support conclusions about the potential toxicity of the new chemical. If the biology of the system affected by a chemical is understood

to a sufficient extent, *in silico* modelling may suffice for hazard and even risk assessment. If such basic information is not available, it may be necessary to employ more complex models to investigate connections between lower and higher levels of biological organisation. In any case, the final results will be subject to quantitative analysis to predict exposure levels that are unlikely to have any adverse effects.

5.4.2 Translation into a New Risk Assessment Framework

One can imagine several different schemes for evaluating chemicals for systemic toxicity. One that has been proposed *(Thomas et al., 2013)* invokes successive tiers of testing with margin-of-exposure as the primary metric. The first tier of the framework integrates a broad range of high-throughput *in vitro* assays such those employed by ToxCast and *in vitro*-to-*in vivo* extrapolation pharmacokinetic modeling. The *in vitro* assays are used to both separate chemicals based on their relative selectivity in interacting with biological targets and identify the concentration at which these interactions occur. The *in vitro*-to-*in vivo* extrapolation modelling converts *in vitro* concentrations into external dose for calculation of the point-of-departure and comparisons to human exposure estimates to yield a margin-of-exposure. In successive tiers, higher level models and other technologies such as transcriptomics are employed to refine the point-of-departure or confirm the mode-of-action. In each tier, the point-of-departure for selective chemicals is based primarily on endpoints associated with a proposed mode of action while the point-of-departure for nonselective chemicals is based on potential generic biological perturbation. The framework provides a risk-based approach to evaluate chemical safety, drawing broadly from previous experience but incorporating technological advances to increase efficiency.

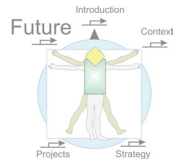
Another possibility is to start with a structure-activity relationship approach, using relational databases for toxicity and chemical structure, and expert rules developed by chemists and toxicologists working together (e.g., *Wu et al., 2010; Blackburn et al., 2011*). The result of this process would be a hypothesis about the action of the new chemical based on the toxicology of its analogues. In some cases the relationship of the analogues will be sufficiently robust to support conclusions about the potential toxicity and potency of the new chemical. In other cases the hypothesis will need to be tested but testing may be guided by the results of the SAR analysis. The testing phase could involve high-throughput or high-content testing, with emphasis on comparison of the results of the new chemical with its analogues. The first scheme is probably better suited for high-throughput applications, the latter for medium-throughput. Other schemas can be envisioned, but all should be based on substantial coverage of the universe of toxicity modes-of-action.

The tools needed to make this vision work will include:

- Cheminformatics, for the development of SAR, QSAR, and a mode-of-action ontology;

- High-throughput assay systems that are constructed to have broad coverage of modes-of-action, for the rapid testing of large numbers of chemicals;
- High-content assays, especially transcriptomics, for more specific information about mode-of-action and to identify where modes-of-action overlap to cause qualitatively different patterns of gene expression (or changes over multiple dosing that may suggest different outcomes versus an acute exposure);
- Systems biology approaches to connect the dots between initial molecular event and outcome, and to support the development of AOPs;
- Higher-order models to support systems biology approaches;
- Pharmacokinetic models to estimate safe dosages *in vivo* from *in vitro* concentrations.

Tools that won't be sufficient are those that only provide a qualitative (yes/no) evaluation of hazard, or an organ-by-organ reconstruction of an organism.



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Glossary

3Rs Reduction, replacement, refinement - defined by Russel & Birch 1959

ADME Absorption, Distribution, Metabolism, and Excretion. ADME describes the disposition of a pharmaceutical compound within an organism (see also TK, toxicokinetics).

ADMET Absorption, Distribution, Metabolism, Excretion, and Toxicity of a compound.

Analogue and / or category approach The terms category approach and analogue approach describe techniques for grouping chemicals. The term analogue approach is used when the grouping is based on a very limited number of chemicals, where trends in properties are not apparent.

A chemical category is a group of chemicals whose physicochemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (or other similarity characteristic). In principle, there should be sufficient members in the chemical category, to enable the detection of trends across endpoints. As the number of chemicals being grouped into a category increases, the potential for developing hypotheses and making generalisations about the trends will also increase, and hence increase the robustness of the evaluation.

AOP An Adverse Outcome Pathway (AOP) describes and formalises the documented, plausible, and testable processes by which a chemical induces molecular perturbations which may lead to a toxic effect. As such it links directly to the associated biological responses which describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal, and population levels of observation. The AOP can then be used to form chemical categories to allow for read across (if appropriate). The AOP can be supported by knowledge of how chemicals interact with biological systems (i.e., the molecular initiating events) and *in vitro* and *in vivo* knowledge of the biological responses.

APAP Acetaminophen (paracetamol), standard reference compound from the **SEURAT-1** Gold Compound list.

API Application Programming Interface: a particular set of commands, functions and protocols that programmers can use to develop software programs that interact with services and resources provided by another particular software program that also implements that API.

AUC Area under the curve

Authentication Confirmation of the identity of a user.

Authorisation Provision of controlled access to resources to a user based on the access permissions they have for the resources.

BAC recombineering A bacterial artificial chromosome (BAC) is a DNA construct used for transforming and cloning in bacteria, usually *Escheria coli*. Recombineering (recombination-mediated genetic

engineering) is a genetic and molecular biology technique that has been developed in *E. coli* and now is expanding to other bacteria species and is used to modify DNA in a precise and simple manner.

BAL Bioartificial liver

BLAST Basic Local Alignment Search Tool

BMD Benchmark Dose: dose levels corresponding to specific response levels, or benchmark responses, near the low end of the observable range of the data. BMDs are obtained from dose-response modelling and can serve as possible points of departure (PODs) for linear or nonlinear extrapolation of health effects data and/or as bases for comparison of dose-response results across studies/chemicals/endpoints.

BMDL A lower one-sided confidence limit on the benchmark dose (BMD).

CAS Chemical Abstract Service

Category formation The process of forming a group of chemicals – often termed a category – on a rational basis, such as having a similar chemical structure or mechanism of action.

Cell Index A dimensionless parameter derived as a relative change in measured electrical impedance to represent cell status.

Cell viability (Equivalent to cell mortality) Number of cells that survives upon a given concentration of a compound.

Chemical category see Analogue and / or category approach.

ChIP Chromatin Immuno-Precipitation, antibody based enrichement analysis of genomic regions to analyse the presence or relative distribution of histone-modifications and histone variants at and across genomic regions

CI Cell Index

Clearance Elimination of a compound by an organ.

CLP Classification, Labelling and Packaging Regulation, i.e. (EC) No 1272/2008.

CNS Central nervous system.

Computational Chemistry Computational chemistry is a discipline using mathematical methods for the calculation of molecular properties or for the simulation of molecular behaviour.

CSR Chemical Safety Report in the context of EU regulations of chemicals (see REACH, CLP)

CSRML Chemical Subgraph Representation Markup Language

CTFA Cosmetic Toiletries and Fragrance Association

CYP Cytochrome-P450

DBD DNA Binding Domain

DEB Dynamic Energy Budget. The theory aims to identify simple quantitative rules for the organization of metabolism of individual organisms that can be understood from basic first principles. The word 'dynamic' refers to the life cycle perspective of the theory, where the budget changes dynamically over time.

DILI Drug-induced liver injury

EB Embryoid body

EC Endothelial cell

EC SCCS European Commission Scientific Committee on Consumer Safety (see entry under 'SCCS')

EC_{50} Half maximal Effective Concentration

ECG Electrocardiogram

ECHA European Chemicals Agency

ECM extracellular matrix

ecopa European Consensus Platform for 3R Alternatives

ENCODE ENCyclopedia Of DNA Elements, NHGRI programme to identify all functional elements in the human genome sequence in the human genome http://genome.ucsc.edu/ENCODE/

ECVAM European Centre for the Validation of Alternative Methods

ER stress Endoplasmatic Reticulum stress

ESC, ES cells See pluripotent stem cells. ES cells are obtained by derivation from the inner cell mass of the embryo at the blastocyst stage (5.5 to 7.5 days after fertilization in the Human).

EST Embryonic stem cell test

ESTIV European Society of Toxicology *In vitro*

Expert system for predicting toxicity This is a broadly used term for any formal system, generally computer-based, which enables a user to obtain rational predictions about the properties or biological activity of chemicals. Expert systems may be classified as knowledge-based (when the rules are based on expert knowledge), induction rule-based (when statistical methods are used to automatically derive the rules) or hybrid (when both approaches are present). One or more databases may additionally be integrated in the system.

FDA U.S. Food and Drug Administration (TG)

FP 7 Seventh Framework Programme for Research and Technological Development of the European Union

fup Fraction unbound to protein

GCCP Good Cell Culture Practice

GDH Glutamate dehydrogenase

Gesicles Methodology for producing proteins and transferring them to target cells, based upon the introduction in producing cells of the gene encoding the viral fusiogenic protein VSVG. Vesicles ("Gesicles" where the G stands for the G viral protein) formed and released by those producing cells are, then, both much more numerous and very prone to fusion with cell membranes. Engineering producing cells with constructs encoding proteins of interest leads to packing of well translated and processed proteins in gesicles, providing a way to produce and transfer proteins into target cells where normal function has been well demonstrated.

GFP Green fluorescent protein

GLP Good laboratory practice

GMP Good manufacturing practice

Gold Compound A well characterised compound for toxicity testing.

GSH Gluthatione

HBV Hepatitis B virus

HCC Hepatocellular carcinoma

hCMC human embryonic stem cell related cardiomyocyte clusters

HCV Hepatitis C virus

HepG2 BAC-GFP A Hep G2 reporter cell line containing the fluorescent moiety (GFP) and a selected gene marker in a Bacterial artificial chromosome (see BAC)

Hep G2cells A HCC derived human hepato-carcinoma cell line (ATCC No. HB-8065) from liver tissue of a 15 year old Caucasian American male with a well differentiated hepatocellular carcinoma.

HepaRG cell line HepaRG is an immortalized cell line of the liver that can be differentiated into hepatocytes which retain many characteristics of primary human hepatocytes.

hES cell Human embryonic stem cell

hiPS cell Human induced pluripotent stem cell

HLC Hepatocyte like cell

HOMO Highest Occupied Molecular Orbital

HSC Hepatic stellate cells

HSEC Hepatic sinusoidal endothelial cells

HTS High-Throughput-Screening

IC10 10% inhibitory concentration

INCI International Nomenclature of Cosmetic Ingredients

***In silico* methods for toxicity prediction** The use of computer-based methods e.g. databases, (Q) SARs, read-across etc to retrieve or estimate toxicological effects of chemicals. These do not require the testing of a chemical (and hence can be termed non-testing information).

Intermediate precursors Cells that are committed to a specific lineage but are not terminally fully differentiated and exhibit the capacity to self-renew without changes in phenotype for a number of passages when grown in culture with specific cocktails of cytokines (e.g. EGF/FGF2 for neural precursors). Intermediate precursors can be terminally differentiated into discrete populations of their lineage. For *SCR&Tox* purposes, intermediate precursor populations are currently available in the neural, mesodermal and keratinocyte lineages

Interoperability The ability of two or more systems or components to exchange information and to correctly use the information that has been exchanged. More generally, it is a property of a system, whose interfaces are completely understood, to work with other systems without any restricted access or implementation.

IPA Ingenuity Pathway Analysis. IPA is a software tool that enables biologists and bioinformaticians to identify the biological mechanisms, pathways, and functions most relevant to their experimental datasets or genes of interest

iPSC, iPS cells See pluripotent stem cells. iPS cells are most commonly obtained nowadays by transferring into replicative donors' cells (e.g. dermic fibroblasts) genes encoding 4 transcription factors (in the original technique, designed by S. Yamanaka, c-Myc, Oct4, Klf4, Sox2). Because current techniques rely on transgene expression, they "alter" cell homeostasis, potentially in a definitive manner. Alternative methods – referred to in the *SCR&Tox* project as "clean reprogrammation" – are therefore actively sought.

IRIS Integrated Risk Information System

iTRAQ Isobaric Tag for Relative and Absolute Quantitation

ITS Integrated Testing Strategy. An ITS is an approach that integrates different types of toxicological data

and information into a decision-making process for the safety of a chemical. In addition to the information from individual assays, test batteries, and/or tiered test schemes, integrated testing strategies may incorporate approaches such as weight-of-evidence and exposure/ population data into the final risk assessment for a substance.

IVIVE *In Vitro* Concentration to *In Vivo* Dose Extrapolation

JNK c-Jun NH(2)-terminal protein kinase pathway

KE Key Events are seminal intermediate events within an Adverse Outcome Pathway that are toxicologically relevant to the apical outcome. They are the basis for hypothesis development and testing and, thus, must be experimentally quantifiable.

KNIME Konstanz Information Miner

Lattice-based model Single-cell based model comprising different classes: (i) each lattice site can be occupied by at most one cell (for cells with homogenous size and shape and fixed positions); (ii) a cell may span many lattice sites (for migrating cells with complex shapes); (iii) lattice sites can be occupied by many cells (for growing cell populations). Lattice models are rule based and do not directly represent the physical reality.

Lattice-free model Represent deformable spheres or ellipses. In some approaches each cell is mimicked by an aggregate of many spheres. Compared with lattice-based models, off-lattice models permit to better directly represent the physical reality.

LBD Ligand Binding Domain

LBP Ligand Binding Pocket

lin-log kinetics Reaction rates are linearly dependent on enzyme concentration and on the logarithm of concentrations. Rates are defined with respect to a reference state.

Linked Data A method of publishing structured data, so that it can be interlinked and become more useful. It builds upon standard Web technologies, but rather than using them to serve web pages for human readers, it extends them to share information in a way that can be read automatically by computers. This enables data from different sources to be connected and queried.

Linked Resources Linked Data approach expanded to all resources including for compounds, biomaterials, assays, algorithms, models, analysis, validation and reports.

LOEL Lowest Observed Effect Level

LSEC Liver sinusoidal endothelial cells

LUMO Lowest Unoccupied Molecular Orbital

LXR Liver X Receptor

MEA Microelectrode array

Mechanism of toxic action The mechanism of toxic action is the molecular sequence of events leading from the absorption of an effective dose of a chemical to the production of a specific toxicological response in the target organ or organism.

MeDIP profile Methylated DNA immuno-precipitation - a method to analyse the DNA methylation across the genome using antibodies directed against modified cytosines (e.g. 5-methylcytosine or 5-hydroxymethylcytosine). Profiling across the genome involved either subsequent next-generation sequencing MeDIP-Seq or array (MeDIP-Chip) technologies.

Meganucleases Endonucleases, either natural or specifically engineered, that are capable of identifying a very discrete region of the DNA and to cut it, resulting in the disruption of a specific sequence with the potential insertion of a construct of interest. One construct used in *SCR&Tox* is a so-called "landing pad", i.e. a sequence that has been engineered in order to facilitate homologous recombination of various gene constructs that will be secondarily introduced into cells that carry the "landing pad". Flanking regions of the "landing pad" have been engineered in order to allow meganucleases to retrieve the entire region, leaving no scar in the host genome.

MID Moulded interconnect device

MIE Molecular Initiating Event, which is the initial point of chemical-biological interaction within the organism that results in a cascade of events leading to an adverse outcome.

miRNA MicroRNA

MoA The Mode of Action relates to the events including, and downstream of, the toxicity pathway. These could lead to an adverse effect in an individual.

MoE The Margin of Exposure is a term used in risk assessment approaches. It is the ratio of the no-observed-adverse-effect level (NOAEL) or the benchmark dose (BMD) to the estimated exposure dose or concentration.

MRM Multiple Reaction Monitoring (MRM), simultaneous quantification of a large number of peptides (several hundreds) in transcriptomics (Toxicoproteomics).

mRNA Messenger RNA

MS Mass spectrometry

M.SssI DNA methyltransferase from Spiroplasma sp. with the DNA sequence specificity CpG.

MTT assay Assays for measuring the activity of enzymes that reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or close dyes (XTT, MTS, WSTs) to formazan dyes, giving a purple color. Used to assess the viability (cell counting) and the proliferation of cells (cell culture assays), as well as cytotoxicity.

NIH reference map Epigenome reference map: A program launched by the NIH to uncover the epigenomic landsacape across human cells
http://www.roadmapepigenomics.org/

NMR Nuclear magnetic resonance

NOAEC No observed adverse effect concentration

NOAEL No observed adverse effect level

NOEL No observed effect level

Non-testing information Non-testing data can be generated by three main approaches: a) grouping approaches, which include read-across and chemical category formation; (quantitative) structure-activity relationships ((Q)SARs); and c) expert systems.

NTP National Toxicological Program

OECD Organisation for Economic Co-operation and Development

OECD Principles for the Validation of (Q)SARs A series of rules to assist in the evaluation of a (Q) SAR for use for regulatory purposes. These state that to facilitate the consideration of a (Q)SAR model for regulatory purposes, it should be associated with the following information:
i) a defined endpoint
ii) an unambiguous algorithm
iii) a defined domain of applicability
iv) appropriate measures of goodness-of-fit, robustness and predictivity
v) a mechanistic interpretation, if possible (COSMOS)

OECD QSAR Application Toolbox Software tool (under development) that allows the user to: a) make (Q)SAR estimations for single chemicals; b) receive summary information on the validation results of the model according to the OECD validation principles; c) receive a list of analogues, together with their (Q) SAR estimates; d) receive estimates for metabolite activation/detoxification information. The Toolbox is freely downloadable from www.qsartoolbox.org

OFAS Office of Food Additive Safety (US FDA)

Ontology An ontology is a formal representation of knowledge as a set of concepts within a domain, and the relationships between those concepts. Domain experts are required to specify an ontology. Computer scientists use ontologies to reason about entities within that domain in the creation of user applications.

PAFA Priority-based Assessment of Food Additives

PBPK models Physiologically-based Pharmacokinetic models. These models apply a realistic mathematical description of physiology and biochemistry to simulate ADME (Absorption, Distribution, Metabolism, Excretion) processes and assess the distribution of chemicals and their metabolites in the

body throughout time. They are particularly adapted to interspecies extrapolation and can be calibrated based on *in vivo*, *in vitro* or *in silico* data.

PBTK Physiologically-Based Toxicokinetics

PCA Principal component analysis

PCPC Personal Care Product Council

PDB Protein Binding Bank

PHCP Personal and household care products

PHH Primary Human Hepatocytes

Pluripotent stem cell lines These cells are of embryonic origin (ES cells) or induced to pluripotency by genetic re-programming of somatic cells from donors (iPS cells). They share two main attributes, unlimited self-renewal –which makes them formally immortal- and pluripotency, the ability to differentiate into any cell type of the body at any stage of differentiation.

PNS Peripheral Nervous System

PoD The Point of Departure is the value on the dose-response curve that serves as the starting point for deriving corresponding health related outcomes (i.e., dose-response for low-dose extrapolation). The POD may be a NOAEL/LOAEL, but ideally is established from BMD modeling of the experimental data, and generally corresponds to a selected estimated low-level of response (e.g., 1 to 10% incidence for a quantal effect). Depending on the mode of action and other available data, some form of extrapolation below the POD may be employed for estimating low-dose risk or the POD may be divided by a series of uncertainty factors to arrive at a reference dose.

Polycomb changes Polycomb proteins are involved in setting and maintenance of epigenetic marks at developmentally regulated genes (such as HOX genes). Changes in the patterns of polycomb genes are indicative of changes in the epigenetic programs set across the genome.

PSCs Pluripotent stem cells

QC Quality control

QIVIVE Quantitative *In Vitro* Concentration to *In Vivo* Dose Extrapolation

qRT-PCR Quantitative real-time polymerase chain reaction

QSAR A Quantitative Structure-Activity Relationship (QSAR) is a quantitative relationship between a biological activity (e.g., toxicity) and one or more molecular descriptors that are used to predict the activity. A molecular descriptor is a structural or physicochemical property of a molecule, or part of a molecule, which specifies a particular characteristic of the molecule and is used as an independent variable in a QSAR.

QT interval The duration of ventricular depolarization and subsequent repolarisation.

RCSB Research Collaboratory for Structural Bioinformatics

REACH Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals.

Read-across A method for filling data gaps in either the analogue or category approaches. Endpoint information for one chemical is used to make a prediction of the endpoint for another chemical, which is considered to be similar in some way. In principle, read-across can be used to assess physicochemical properties, environmental fate and (eco)toxicity effects, and it may be performed in a qualitative or quantitative manner.
In qualitative read-across, the potential of a chemical to exhibit a property is inferred from the established potential of one or more analogues.
In quantitative read-across, the numerical value of a property (or potency of an endpoint) of a chemical is inferred from the quantitative data of one or more analogues.

RMCE Recombinase-mediated cassette exchange. RMCE is of increasing interest in the field of reverse genetics. The procedure permits the systematic, repeated modification of higher eukaryotic genomes by targeted integration. In case of RMCE, this is achieved by the clean exchange of a pre-existing 'gene cassette' for an analogous cassette carrying the 'gene of interest'.

RNA Ribonucleic acid

ROS Reactive Oxygen Species

RPTEC/TERT1 Human renal proximal tubular cell line, immortalized by hTERT transfection

RT-CESTM Real-Time Cell Electronic Sensing

RTD Research and technical development

RXR Retinoid X Receptor

SAR Structure Activity Relationships (SARs) are theoretical models that can be used to predict in a qualitative manner the physicochemical, biological (e.g., toxicological) and fate properties of molecules from knowledge of chemical structure. More specifically, a SAR is a qualitative relationship (i.e. association) between a molecular (sub)structure and the presence or absence of a given biological activity, or the capacity to modulate a biological activity imparted by another substructure.
The term substructure refers to an atom, or group of adjacently connected atoms, in a molecule. A substructure associated with the presence of a biological activity is sometimes called a structural alert.
A SAR can also be based on the ensemble of steric and electronic features considered necessary to ensure the intermolecular interaction with a specific biological target molecule, which results in the manifestation of a specific biological effect. In this case, the SAR is sometimes called a 3D SAR or pharmacophore.

SAX Strong anion exchange fractionation technique

SCCS Scientific Committee on Consumer Safety. This EU Committee provides opinions on health and

safety risks (chemical, biological, mechanical and other physical risks) of non-food consumer products (e.g. cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products) and services (e.g. tattooing, artificial sun tanning).

SEP Scientific Expert Panel of the **SEURAT-1** Research Initiative. The SEP provides scientific advice regarding the research work and future orientation of **SEURAT-1**.

shRNA Short hairpin RNA

siRNA Short interfering RNA

SMARTS A language in Computational Chemistry for describing molecular patterns.

SOP Standard Operating Procedure

SQL Often referred to as 'Structured Query Language' is a programming language designed for data management.

SREBP-1c Sterol Regulatory Element-Binding Protein 1c

Tanimoto criteria Molecular similarity criteria for chemicals based upon Tanimoto Coefficients.

TBBB The ToxBank BioBank (TBBB) will establish a banking information resource for access to qualified cells, cell lines (including stem cells and stem cell lines), tissues and reference materials to be used for *in vitro* predictive toxicology research and testing activities.

TBCR The ToxBank Chemical Repository will ensure the availability of test compounds to researchers of the **SEURAT-1** Research Initiative.

TBDW The ToxBank Data Warehouse will establish a centralised compilation of data for systemic toxicity.

TBGCD The ToxBank Gold Compound Database will provide a information resource servicing the selection and use of test compounds.

TD Toxicodynamics, the processes and interactions of an exogenous compound within an organism, including the compound's effects on processes at the organ, cellular, and molecular levels.

TG-Gates Data-base of the Japanese Toxicogenomics project - Genomics assisted toxicity evaluation system (http://toxico.nibio.go.jp/english/index.html).

TK Toxicokinetics, the processes by which a substance reaches its target site. This includes absorption (the process of a substance entering the organism), distribution (the dispersion of substances throughout the fluids and tissues of the organism), metabolism (the irreversible transformation of substances by the organism), and excretion (the elimination of substances from the organism. These four processes are also referred to as ADME.

Toxicity Pathway A toxicity pathway is a cellular response pathway that, when sufficiently perturbed, is expected to result in adverse health effects.

Toxicological data Data relating to the harmful (toxicological) effects of chemicals. This may include information from animal, human or non-animal (*in vitro*) tests.

TTC Thresholds of toxicological concern (TTCs) have been developed for risk assessment of compounds of known chemical structure for which no compound-specific toxicity data are available. Below the TTC value the risk to human health is assumed to be negligible. The TTC may be used as a substitute for substance-specific information in situations where there is limited or no information on the toxicity of a compound, and where human exposure is so low, i.e. below the corresponding TTC, that adverse effects are not to be expected.

UPR Unfolded protein response pathway

US FDA United States Food and Drug Administration

US EPA United States Environmental Protection Agency

VE-cadherin Vascular endothelial cadherin

Web Service A method of communication between two electronic devices over a network.

ZFN-HR Zinc finger nuclease homologous recombination

Dr José-Manuel Zaldívar Comenges

(1958–2012)

José-Manuel Zaldívar Comenges, known to his colleagues as Josema, was a senior scientist at the European Commission's Joint Research Centre (JRC). He died on 14 September 2012, after a period of ill health.

Josema joined the JRC in March 1987, with an MSc in Organic Chemistry from the Universidad Autònoma de Barcelona, and a PhD in Chemical Engineering from the Universiteit Twente, The Netherlands. A brilliant scientist, Josema dedicated his career to the application of advanced mathematical modelling to a wide and diverse range of problems, including the computer modelling of chess games, the management of traffic flow in Barcelona, the safety assessment of chemical reactors, and the modelling of chemical fate in geochemical, ecological and biological systems. He was a prolific writer and innovator, with more than 300 scientific reports and peer review publications, two patents and five software copyrights to his name. His extensive and varied activities have not only contributed greatly to the development of scientific methodology, but have resulted in numerous applications for the protection of human health and the environment. He joined the JRC's Institute for Health and Consumer Protection (IHCP) in 2007, where he turned his mathematical expertise to chemical toxicology, playing a leading role in the development of chemical risk assessment methodology, and establishing a new activity on the application of systems biology to the modelling and assessment of chemical toxicity. Josema's considerable accomplishments have been recognised by both the JRC and the international scientific community.

Josema is remembered by his colleagues with great affection and appreciation – affection for a wonderfully kind and generous man, with a great sense of humour and occasion; and appreciation for his dedication, insights and collegial manner. Outside of work, Josema was devoted to his family, his wife Fernanda, and his two daughters, Giulia and Sara.

In the words of his wife, Fernanda, *"Josema loved life, his family and his work and taught others to do the same. When you worked with him you had the feeling that everything would be successful and that the work would have been a great success."* Indeed, thanks to Josema's inspiration and scientific legacy, researchers at the JRC continue to follow his vision, and the young scientists he trained are already enjoying their own successes and scientific recognition.

Andrew Worth
European Commission
Joint Research Centre
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Systems Toxicology Unit

Gabrielle M Hawksworth

(1947–2012)

Professor Molecular Toxicology, University of Aberdeen, Co-Chair of the **SEURAT-1** SEP

I first met Gay when I was an impressionable PhD student at St Mary's Hospital Medical School, from where she herself had graduated with a PhD under one of the fathers of modern xenobiotic metabolism and biochemical toxicology, Tecwyn Williams. It was the occasion a UK Drug Metabolism Workshop and, amidst all the talk of TLC plates, rat urine samples and chemical structures, I was fascinated by a presentation which Gay gave. She didn't just talk about metabolism for its own sake, but introduced the words "mechanism of toxicity" as well, and talked of translational risks to man. This was quite a turning point for me personally and was to greatly influence my own career in toxicology research and teaching, as I followed the path she helped to lay for me!

Gay was one of those scientists who moved between the seams of different disciplines, sewing the fabric through careful bench experimentation and close fostering of her students, of which she mentored over 30 individual PhDs. Her training in pharmacology and insight into its relevance to human toxicity has left a number of major legacies in the service of the public. From founding seminal courses such as the MSc course in Clinical Pharmacology at Aberdeen in 1982, to her publication of over 150 major peer reviewed articles in biochemical and molecular toxicology, Gay worked tirelessly to provide quality teaching and mentorship, research tools and scientific knowledge to improve the way in which we risk assess chemical hazards in our society. Gay also worked energetically with several of our professional socities, being past General Secretary and President of the British Toxicology Society (BTS) and a council member of the International Society for the Study of Xenobiotics (ISSX). As a scientific advisor, Gay was also prominent in a number of UK Government bodies, notably the influential Committee for Pesticides.

At its conception, the **SEURAT-1** Research Initiative was an ambitious one, aiming to provide an experimental framework for the development of chronic toxicity models without using experimental animals. This seemingly tall order was further compounded by the long-term aim of providing a testing-framework that facilitates human safety assessment, based wholly on a 'mode-of-action' paradigm. In clustering the 6 individual consortia into a research cluster, a Scientific Experts Panel (SEP) was formed and Gay was a natural choice for its Co-chairmanship. Her sharp intellect, straight talking style and warmth in meeting with others not only functioned to foster the scientific direction of the **SEURAT-1** Research Initiative, but also greatly facilitated the difficult conversations which were necessary in the founding period. She stood for knowledge, experience and vision, which all members of the cluster could relate to and respect.

To quote Eleanor Roosevelt "You rarely achieve finality. If you did, life would be over. But as you strive new visions open before you, new possibilities for the satisfaction of living". Gay Hawksworths life and works have left their mark on many of us, but for the members of the **SEURAT-1** Research Initiative her vision and wisdom are deeply engrained in its fabric and she will always be associated with the successes to come.

Ian Cotgreave
*On behalf of all **SEURAT-1** SEP and Cluster members*

This book is prepared by the Coordinating Action COACH team, consisting of the Scientific Secretariat and the Scientific Expert Panel (SEP)* within the SEURAT-1 Research Initiative

COACH: Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals (Grant agreement N° 267044)

The COACH scientific secretariat

ARTTIC

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* A detailed description about the role of the Scientific Expert Panel including information about the members can be found in the Introduction

- On 11 March 2013 the full ban on animal testing for cosmetic products came into force. From this date the marketing of new cosmetic products tested on animals in the European Union is prohibited in accordance with the seventh amendment of the 'Council Directive on the approximation of the laws of the Member States relating to cosmetic products' (7677687EEC9). The European Commission, together with Cosmetics Europe, launched the Research Initiative 'Towards the replacement of *in vivo* repeated dose systemic toxicity testing' in order to develop a sound research strategy leading to the long-term target of 'Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT). This Research Initiative is called **SEURAT-1** and comprises six research projects focusing on the development of new test methods in the field of repeated dose systemic toxicity.

- This is the third volume in a series of six annual reports that will, step by step, pave the way towards innovative safety evaluation of chemicals in various fields of application (for example, medicine, personal care, agriculture, food production, ingredients of everyday products).

- The specific goal of this Research Initiative is the development of *in vitro* test systems based on human cell lineages and related *in silico* methods, which is considered to be a first step towards the replacement of *in vivo* repeated dose systemic toxicity testing. **SEURAT-1** will bring the long-term research target to the proof-of-concept stage.

The purpose of the book is:

to inform policymakers about scientific progress relevant to the implementation of European Directives and Regulations, fully respecting the 3Rs-principle;

to inform research policymakers about essential gaps in knowledge and corresponding research needs;

to open a dialogue with regulatory authorities to update current legislation in line with scientific progress;

to support industry in the implementation of the most advanced test methods, thereby increasing their competitiveness;

to encourage the extension of the Research Initiative activities at national, European and international levels.