EUROPEAN COMMISSION
Research & Innovation

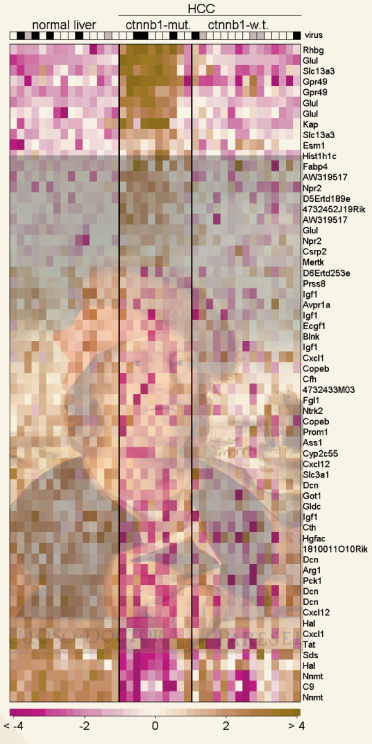
Cosmetics Europe
the personal care association

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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SEVENTH FRAMEWORK PROGRAMME

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"

The Proof-of-Concept Case Studies

Edited by:
Tilman Gocht, Michael Schwarz

volume 4

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** develops 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

In January 2014, the **SEURAT-1** Research Initiative completed its 3rd year of existence and went through a thorough review by independent experts. Recognising that the scientific and organisational targets of this research initiative, co-funded by the European Commission and Cosmetics Europe, are highly challenging, the reviewers noted tangible successes and important achievements of **SEURAT-1** along the path to developing non-animal methods for the replacement of *in vivo* repeated dose systemic toxicity testing.

The individual projects of the **SEURAT-1** Research Initiative have made good progress in a number of areas including the establishment of well characterised stem cell-derived *in vitro* models and high throughput '-omics' assays for the identification of toxicity pathways, the development of complex bioreactors, the generation of a database for cosmetics and the collection of data in a dedicated warehouse.

Collaboration within the cluster has gained momentum and it is time to start applying the knowledge generated to establish test methods that could ultimately receive regulatory approval. Accordingly, **SEURAT-1** has initiated an important proof-of-concept exercise, based on three-layered case studies, to demonstrate the potential for safety assessment of the tools and knowledge generated through the individual projects. This exercise might also lead to the identification of gaps in safety assessment and indicate where future research efforts should concentrate.

Last year, **SEURAT-1** explored the possibility to collaborate with the Tox21 research programme in the USA that develops high throughput tools to prioritise thousands of chemicals for toxicity testing. Such international collaborations with complementary initiatives should certainly be strengthened and extended in the future in order to address the global challenge to deliver new and better safety assessment approaches.

January 2014 was also the official start date of Horizon 2020, the European Union's new Framework Programme for Research and Innovation, which will run for 7 years until 2020. This programme will provide further opportunities to support research into non-animal approaches to predictive safety testing through different dedicated topics in annual calls, including topic PHC 33, 'New approaches to improve predictive human safety testing' in the 'societal challenge 1: *Health, demographic change & wellbeing*'. With an available budget of €30 million and a deadline for proposals expected in early 2015, one or more funded projects can be anticipated. Projects should capitalise on advances in all relevant fields of science to understand complex biological pathways of toxicological relevance and to identify early markers predictive of toxicological effects in humans with the objectives of developing and validating routine, non-animal approaches for toxicity testing of chemical substances. The scope of topic PHC 33 is quite broad, as the project(s) should include not only research communities, but also SMEs, industry and regulatory agencies as appropriate. In addition international collaboration with similar initiatives in the USA and elsewhere is encouraged. Finally, the Joint Research Centre from the European Commission intends to collaborate with all selected projects.

Overall, **SEURAT-1** has already delivered some laudable results and established itself as a flagship activity in the field. We wish all the **SEURAT-1** participants success in the one and a half years remaining, in fully and jointly working to achieve their challenging goal to lay down the foundation for new innovative knowledge-based approaches to safety assessment.

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Executive Summary

On 11 March 2013 the full ban on animal testing for cosmetic products within the European Union entered into force. 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 fourth 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 HEALTH programme of the Seventh European Research Programme (FP7) in 2008. The framework for this Research Initiative was created in June 2009 following 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 through the HEALTH programme of 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. This experimental work is linked with advanced methods of computational modelling and estimation techniques, taking innovative systems biology approaches into consideration; this requires 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 integrated animal-free prediction 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. The roadmap was created by the coordination and support action project (COACH) in close cooperation with the project coordinators, and was 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.

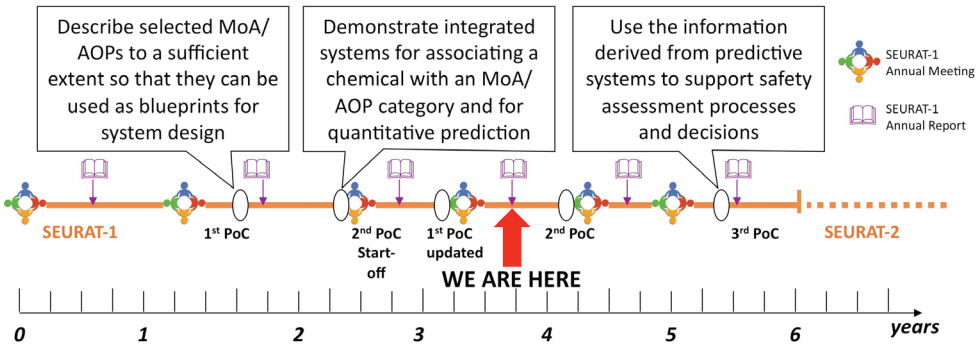

Figure 1 *Roadmap illustrating the timing of the proof-of-concept (PoC) at three conceptual levels as the backbone for interactions between the* ***SEURAT-1*** *projects.*

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) data exchange between 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 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 fourth Annual Report, prepared by COACH, presents: (i) a comprehensive overview of research highlights from the different projects of the **SEURAT-1** Research Initiative; and (ii) descriptions of the proof-of-concept case studies at the three above-mentioned levels. As shown in *Figure 1*, the focus is currently on the level of test system development based on already existing mode-of-action descriptions. In addition, planning of the case studies at the application level is ongoing and two different scenarios were developed, to which methods developed within **SEURAT-1** will contribute (see below). Altogether, this fourth Annual Report marks a transition from the development of the **SEURAT-1** research strategy and proofs-of-concept at the cluster level (first three volumes) to the concrete demonstration of how **SEURAT-1** will ultimately support safety assessment through results of the proof-of-concept case studies facilitated by the innovative toolbox provided by the **SEURAT-1** projects. Beyond **SEURAT-1**, the case studies will also provide guidance towards further development of mechanism-based integrated toxicity testing strategies and modern safety assessment approaches.

The Annual Report is organised in five chapters: chapter 1 provides a general introduction to

the **SEURAT-1** Research Initiative. It describes the model of **SEURAT-1** as a cluster of projects in the context of the call for research proposals under FP7. Furthermore, it introduces the cluster-level objectives as well as the structure and organisation of the **SEURAT-1** Research Initiative. Finally, some key elements of the new European Framework Programme for Research and Innovation (Horizon 2020) are highlighted with a focus on possible **SEURAT-1** follow-up activities.

Chapter 2 outlines the context of the **SEURAT-1** Research Initiative from the following perspectives:

(i) Legislation: On 11 March 2013 the full ban on animal testing for cosmetic products in the EU came into force, despite the fact that alternative methods to animal testing were not available for a number of endpoints. The history and rationale of the testing ban on cosmetic products, as well as the consequences of its implementation, was thoroughly discussed in the first three volumes of this Annual Report and is not further addressed in this fourth volume.

(ii) Regulation: The clear focus this year is on the regulatory risk assessment context. Now that animal testing for cosmetic ingredients is banned, pressure has increased on both scientific efforts to develop animal-free testing strategies and regulatory implementation of such methods into safety assessment. Moving away from animal testing reformulates the question of how to deal with uncertainties; a regulatory perspective on integrating non-standard data into hazard and risk assessment is described in this volume, differentiating between uncertainties due to lack of knowledge and uncertainty arising from variability and inherent randomness. Another important issue when implementing new methods is how to validate them, i.e., to assess a method's reproducibility and relevance for a given purpose. These questions are addressed in another section of this chapter, which outlines a science-based approach, termed 'mechanistic validation', for validating animal-free toxicity testing methods. It should be noted that the **SEURAT-1** proof-of-concept case study approach, thoroughly discussed in chapter 3, follows the same line of thinking as the concept of 'mechanistic validation'. Finally, a pragmatic complementary approach to safety assessment of low-exposure chemicals without new toxicity testing is described in this section; i.e. the concept of the Threshold of Toxicological Concern (TTC). This concept is based on the availability of reliable exposure information and existing toxicity data. At the heart of this approach, two components are central: a toxicity database (with a clear method to determine the point of departure) and a chemistry tool (decision tree) used to classify chemicals into potency classes. Initially developed for food additives, the applicability domain of this approach is currently being extended to other substances including cosmetic

ingredients, taking into account different exposure routes (such as oral-to-dermal extrapolation).

(iii) Science: This year's Annual Report provides an overview of toxicological endpoints in the chemical space of cosmetics, where accepted non-animal test methods are still lacking. Cosmetics Europe developed a research and science programme to further advance the field of method development, which is described in a separate section. In addition, taking into account the current demands from industry to apply non-animal methods in the context of safety assessment, a separate section discusses 'read-across'. This is a concept that can be used for data-rich chemicals with well-established toxicological profiles to predict the toxicity of structurally-related chemicals that lack toxicity data, either as one-to-one read-across or within a group (or category) of similar chemicals. Information from *in vitro* molecular screening, '-omics' assays and computational models can be used to improve the robustness of the read-across case. Finding appropriate groups of chemicals to demonstrate the feasibility of this approach is scientifically challenging, and scenarios for read-across case studies were intensively discussed at a **SEURAT-1** workshop in April 2014 in Ispra, Italy, and summarised in an expert report. The scenarios refined and examined by the workshop participants were: (i) chemical similarity of direct-acting toxicants with a similar mechanism of action (no metabolism or metabolism not a driver of toxicity); (ii) chemical similarity involving metabolism-driven toxicity (resulting from exposure to parent toxicants with similar metabolites); (iii) chemical similarity of toxicants with no obvious reactive or specific mode-of-action (generic effects of low potency); and (iv) chemical similarity of toxicants with overt toxicity and a presumed mode-of-action. The chemical selection strategy for these scenarios completes the second chapter of this Annual Report, introducing aspects of the **SEURAT-1** proof-of-concept case studies that are described in detail in chapter 3.

In the previous volumes, chapter 3 focused on the development of a long-term research strategy and its implementation within the cluster. 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. As already mentioned above, the theoretical work is considered 'completed' and the challenge is now to convert this into praxis by means of the formulation of proof-of-concept case studies. These case studies should demonstrate that knowledge about modes-of-action can be converted into the development of integrated testing strategies to be used for safety assessment. This fourth Annual Report outlines the steps for designing these testing strategies as well as brief descriptions of each of the case studies.

Text box: The **SEURAT-1** Proof-of-Concept Case Studies
Knowledge Level: Level 1 Proof-of-Concept Case Studies Challenging the predictive power and robustness of an AOP construct from bile salt export pump inhibition to cholestatic injury Available Mode-of-Action Descriptions: From protein alkylation to liver fibrosis From liver X receptor activation to liver steatosis From inhibition of the bile salt export pump to cholestasis
Methodological Level: Level 2 Proof-of-Concept Case Studies Investigation of the fibrotic response induced by methotrexate and acetaminophen in the *HeMiBio* bioreactor Evaluation of valproic acid induced steatosis in HepaRG cells Use of biomarkers to substantiate the read across prediction Screening of perturbed toxicity pathways by transcriptomics fingerprinting of data poor substances Developing chemotypes for mitochondrial toxicity Mode-of-action-based classification model for repeated dose liver toxicity
Application Level: Level 3 Proof-of-Concept Case Studies Read-across using **SEURAT-1** evidence *Ab initio* case study

In principle, the first proof-of-concept level has already been achieved within **SEURAT-1** by the development of the three theoretical adverse outcome pathways (AOPs) for the three major liver adverse outcomes, which are fibrosis, steatosis and cholestasis. These are now taken as the foundation for the development of integrated testing strategies, i.e. the level 2 proof-of-concept case studies. Test systems focus on certain key events and their sensitivity and specificity will be assessed by a sophisticated selection of standard reference compounds demonstrating that the test system is indeed predictive for the mechanism addressed (which follows a strategy of 'mechanistic validation'). Alternatively, AOP knowledge can be applied when choosing a key event common for many pathways, and then predict general toxicity affecting many organs simultaneously (for example, mitochondrial toxicity). This is also reflected in the level 2 proof-of-concept case studies. Furthermore, a flexible 'conceptual framework' has emerged from **SEURAT-1** 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 in the context of repeated-dose systemic toxicity. This is used for the level 3 case studies at the application level. These reflect two typical safety assessment scenarios: (i) the objective of the first case study is to arrive at a point of departure

for a particular chemical, that 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, that was mentioned earlier, is to use the **SEURAT-1** methods in the context of 'read-across', that is, to demonstrate that information using **SEURAT-1** methods can be used to improve the validity of a 'read-across' justification so that toxicological properties from tested source substance(s) can be 'read across' to 'target' substance(s) within a chemical category. The definition and execution of the case studies on all three proof-of-concept levels 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.

The detailed project descriptions and their research highlights from the third 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 and highlights of each of the projects 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 development of pluripotent stem cell-derived neuronal models for toxicity testing. A differentiation protocol was developed and the stem-cell-derived neuronal cells were thoroughly characterised and compared with a benchmarking neuronal cell model. The new stem-cell derived neuronal cell model is now being used in a repeated dose toxicity study.

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

HeMiBio reports on progress in the characterisation of hepatic stellate cells. Mechanisms controlling stellate cell activation were studied and the first gene and microRNA expression profiles as well as the first epigenetic pattern in human purified and uncultured liver cell types indicative for stellate cell activation were obtained. Furthermore, a prototype for a 3D flow-over bioreactor was developed and permits the continuous monitoring of cell viability for over 28 days *in vitro* and a high-resolution analysis of hepatotoxicity.

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

The DETECTIVE report focuses on human skin-derived precursors as a novel cell source for evaluating the hepatotoxic potential of chemicals. Using

a differentiation protocol based on hepatogenic growth factors skin-derived precursors acquired features of hepatic progenitor cells. Toxicogenomic analysis revealed that these cells respond to acetaminophen exposure in a comparable way to primary human hepatocytes in culture. Commonly upregulated genes might represent potential molecular biomarkers for hepatic toxicity.

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 released the first version of the COSMOS database in December 2013. It is a chemo-centric system which provides chemical and toxicological data to support the data needs of **SEURAT-1** partners, as well as safety assessors in public and private organisations. The data quality was assessed and the database supports data retrieval via a user-friendly web interface which allows querying by chemical, toxicological or both types of data, as well as grouping within an AOP framework. A second key aspect of COSMOS activities in the third year was the evaluation of the applicability of the TTC approach to cosmetics. A non-cancer database was curated for this purpose and a tiered decision-tree approach has been developed as a guide to estimate systemic bioavailability following dermal exposure to cosmetics when applying the oral TTC in the absence of toxicity data.

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 reports on the *in vitro* cultivation of organotypic HepaRG cultures for long-term repeated dose toxicity studies. Different culture conditions were tested and their effects on the intracellular fluxes were determined by metabolic flux analysis. Serum-free and serum-supplemented conditions are suitable for long-term cultivation (up to 30 days) of 2D cultures and 3D spheroids. 3D organotypic co-cultures were also characterised. Based on these results, long-term repeated dose toxicity screening studies were conducted for selected compounds. The oral equivalent dose concept was applied for *in vitro* to *in vivo* extrapolation.

ToxBank

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

The ToxBank report focuses on integrated '-omics' analysis of the

SEURAT-1 standard reference compounds ('Gold Compounds'). New tools were implemented in the ToxBank Data Warehouse that allow the easy export of raw and processed data, enabling integration with general bioinformatics tools for analysis and enrichment along with analysis, visualisation, modelling, and data mining tools to support the understanding of the results and performance of data meta-analyses. In addition, precise searching for chemical structures has been added to the ToxBank Data Warehouse (exact, substructure, and similarity) to support read-across and information look-up.

COACH

Cluster-level coordination and support action.

The COACH report provides information about the cluster-level coordination activities, the facilitation of 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 overview 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. 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 of the **SEURAT-1** Research Initiative. The list of short project descriptions that was included in the previous Annual Reports has been further updated, with special emphasis on initiatives focusing on repeated dose toxicity and the replacement of animal testing in the field of human safety assessment. Existing and envisaged collaborations between **SEURAT-1** partners and these various related international activities were highlighted, underlining the integration of **SEURAT-1** into the field. Indeed, 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'. Accordingly, an important event was the workshop entitled '*SEURAT-1 meets Tox21'*, which was conducted in June 2013. Principal scientists from both initiatives were brought together to discuss opportunities for cooperation. The key outcome of the information exchange and discussion was a comprehensive list of cooperation topics at both the technical level, including the sharing of research materials, such as data, cells, assays, computational models, and at the application level, by teaming up on predictive toxicity and safety assessment proof-of-concept case studies.

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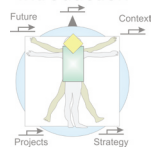


1 INTRODUCTION

Tilman Gocht, Michael Schwarz

'Coming together is a beginning; keeping together is progress; working together is success.'
Henry Ford

Background

On 11 March 2013 the full ban on animal testing for cosmetic products within the European Union entered 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. This deadline was not further extended, even though 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; *Adler et al., 2011*),

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.

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.

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 and identifying gaps in knowledge, which are to be bridged by future research work. The end result will be *in vitro* testing methods and *in silico* tools 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 contributes 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 from 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*) published in the third volume of this book series. Relevant case studies addressing these three proof-of-concept levels were formulated in the last year and will be discussed in this fourth volume. 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.

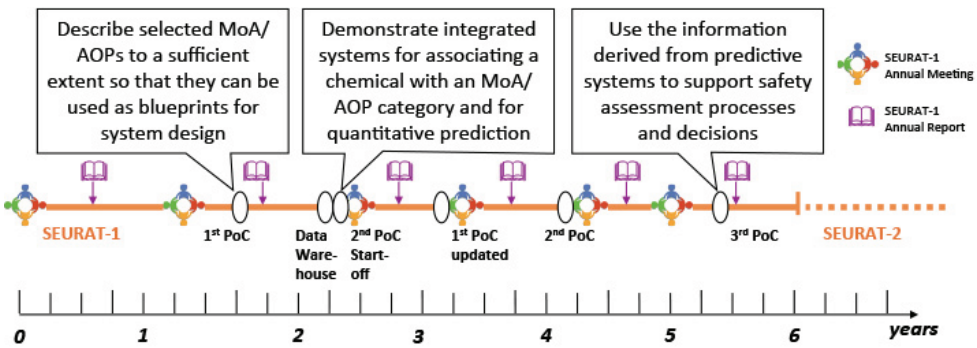


Figure 1.1 SEURAT-1 *roadmap illustrating the timing of the proof of concept (PoC) at three conceptual levels as the backbone for interactions between the* ***SEURAT-1*** *projects.*

The Annual Report: Something about 'Pathways'

This is the fourth 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 following 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 context 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 fourth volume we specify the cluster-level case studies that will combine theoretical mode-of-action descriptions with integrated testing strategies, demonstrating how test systems can be produced by integrating various *in vitro* and *in silico* tools emanating from the **SEURAT-1** projects, in order to assess the toxicological properties of chemicals using modes-of-action as an analytical basis. Further case studies will address the desire to show how the data and information derived from the tools and methods developed within **SEURAT-1** can be used in specific safety assessment frameworks and scenarios. This chapter is followed by detailed project descriptions in chapter 4, which provides an overview of research highlights from the past year. Finally, chapter 5 focuses on related international activities and identifies potential interfaces for establishing collaborations on 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.

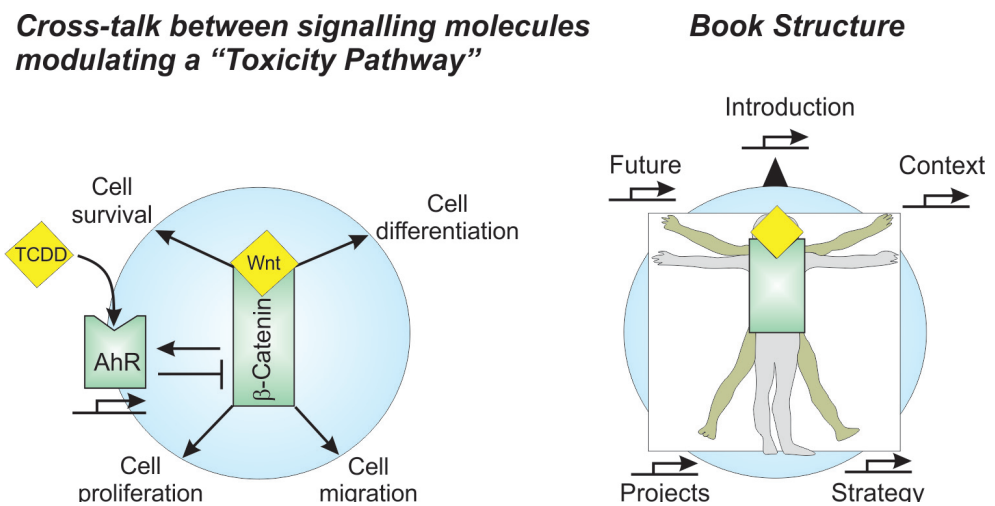
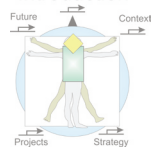


Figure 1.2 *The concept of 'Toxicity Pathways' (left panel) is mirrored by the book structure (right panel). Toxicity pathways may include cell-cell-interactions. Here we exemplify this process (by showing the effect of a chemical on gap junctional intercellular communication (GJIC, left panel).*



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, material scientists 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) advises 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 and 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 / Country	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**	
George Daston	Procter & Gamble, Product Safety and Regulatory Affairs, Cincinnati / USA	
Derek Knight	**European Chemicals Agency, Helsinki / Finland**	
Catherine Mahony	Cosmetics Europe (Procter & Gamble), London Innovation Centre / UK	
Russell Thomas	National Center for Computational Toxicology, U.S. Environmental Protection Agency, Research Triangle Park / USA	

Recent Developments: Human Safety Assessment and Horizon 2020

The continuation of the SEURAT programme will be possible under the umbrella of the European Commission's new funding scheme, Horizon 2020, which will make a total of €80 billion in funding available between 2014 and 2020. The theme 'Health, demographic changes and wellbeing' was identified as one of six societal challenges on which funding will be focused. The Work Programme for the years 2014–2015 highlights 'personalising health and care' as the particular area of interest, in which 34 topics among 7 focus areas will be funded with a total of €1.21 billion (*European Commission, 2013*). The most relevant call for proposals for **SEURAT-1** activities, entitled 'New approaches to improve predictive human safety testing' (call identifier PHC-33-2015) can be expected in the area 'Improving health information, data exploitation and providing an evidence base for health policies and regulation' (a draft version can be found at http://ec.europa.eu/research/participants/portal/desktop/en/opportunities/h2020/topics/2283-phc-33-2015.html). The objective is to develop and validate routine, non-animal approaches for toxicity testing of chemicals by means of mechanism-based understanding of complex biological pathways of toxicological relevance and identification of early markers predictive of toxicological effects in humans. The relationship of this new project with the scope of the **SEURAT-1** Research Initiative is obvious and further calls for proposals from other areas of societal challenges (e.g., SFS 12–2014 'Assessing the health risks of combined human exposure to multiple food-related toxic substances' with the scope to reduce the use of animals in toxicological research, published within the societal challenge 'Sustainable food security') underline the importance of starting additional joint efforts to accelerate the paradigm shift in toxicity testing from empirical *in vivo* studies to mechanism-based approaches combining *in vitro* tests with *in silico* methods.

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2 CONTEXT

'Ethics and Science need to shake hands.'

Richard Clarke Cabot

2.1 Introduction

Tilman Gocht, Michael Schwarz

The Seventh Amendment to the Cosmetics Directive introduced a number of key requirements related to animal testing, that were incorporated into the Cosmetics Regulation (Regulation (EC) No 1223/2009, 30 November 2009). In 2004 the testing of cosmetic products on animals was banned within the EU. In 2009 a 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/sub-chronic 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.

This chapter is intended to outline the recent developments in the legal, regulatory and scientific context of the **SEURAT-1** Research Initiative. This year the chapter focusses on questions arising from the implementation of innovative approaches using non-animal data into regulation. It starts with a contribution outlining the toxicological endpoints in the chemical space of cosmetics, where accepted non-animal test methods are still lacking. The current and future efforts of Cosmetics Europe to further advance the field of method development to close these gaps are highlighted here. Secondly, it introduces the concept of the Threshold of Toxicological Concern, which allows, in the case of small amounts, a preliminary risk assessment of chemicals without any toxicity testing based on the availability of reliable exposure information alone. In any safety assessment strategy uncertainties must be addressed: a quantitative assessment of a toxicological endpoint requires a quantitative assessment of the respective uncertainty (independent of whether animal data or *in vitro* and *in silico* data are being used in the assessment). Moving away from animal testing may reformulate the questions regarding uncertainties and therefore the third contribution in this chapter describes the regulatory perspective on demands arising from integrating non-standard data into safety assessment. Another important question when implementing new methods into regulation is how to validate these new methods, *i.e.* how to independently assess the reproducibility and relevance of a test for a given purpose. These questions are addressed in the fourth section, which outlines a science-based approach for validating animal-free toxicity testing methods; this approach is termed ‘mechanistic validation’. The **SEURAT-1** proof-of-concept case study approach, thoroughly discussed in chapter 3, follows the same line of thinking as the concept of ‘mechanistic validation’. Finally, apart from developing new toxicity testing methods and implementing into regulation, there is a strong need for industry to apply such methods now in the context of safety assessment. The most obvious way of doing so is to perform read-across exercises, demonstrating that

data-rich chemicals with well-known toxicological profiles can be used to predict the toxicity of chemicals that lack toxicity data, provided that both belong to the same group or category of chemicals. Scenarios for such read-across case studies were intensively discussed at a **SEURAT-1** workshop in April 2014 in Ispra, Italy, and these scenarios are presented in the last section of this chapter, introducing aspects of the **SEURAT-1** proof-of-concept case studies that are described in detail in chapter 3.

2.2 Cosmetics Europe's Research Initiative Following the Testing Ban

Patric Amcoff, Rob Taalman

2.2.1 Introduction

Cosmetics Europe - The Personal Care Association has been the voice of Europe's cosmetic, toiletry and perfumery industry since 1962 and represents the interests of more than 4000 companies. In 2013, direct and indirect employment in the European cosmetics industry was approximately 1.7 million people including 25.000 scientists. Every cosmetics product on the market in Europe is safe to use. The cosmetics industry can state this with confidence because first, safety is the primary concern of all manufacturers, and secondly, European Union legislation requires all new products to undergo an expert scientific safety assessment before they are launched for sale.

While safety for the consumer is the industry's prime concern, science and innovation are its drivers. Innovation is crucial because most cosmetics products have a lifespan of less than five years and manufacturers reformulate a quarter of their products every year. They need to improve products constantly in order to stay ahead in a highly competitive market where the consumer expects more choice and ever greater efficacy. Scientific research and development is essential to the cosmetics industry. It can take several years to bring a product to market and safety is built in at every stage in the process.

For more than 20 years the cosmetics industry's best scientists have been dedicated to successfully developing alternative approaches to animal safety testing. Cosmetics Europe plays a leading role in supporting the development, scientific and political acceptance and finally the use of alternative testing methods and is dedicated to meet the challenges lying ahead by close collaborations and partnerships.

2.2.2 Implications of the Testing Ban

The animal testing/marketing ban has been in place for more than one year (implementation date March 2013; *European Commission, 2013a*), however, it is difficult to judge the impact at this point in time given that the consumer market is influenced by many factors including new legislation. Any new regulation presents a challenge but the testing/marketing ban puts a special burden on the Cosmetics industry in Europe to remain innovative.

Prior to the implementation of the 2013 marketing ban the EU Commission had to establish to which extent alternative methods for testing cosmetic products and their ingredients for the relevant endpoints are available by 2013. The conclusions from this exercise were that alternative replacement test methods have not yet been developed or accepted for regulatory safety assessment for testing of acute toxicity, reproductive toxicity, carcinogenicity/mutagenicity, skin sensitization/photosensitization or repeat-dose toxicity (*Adler et al., 2011*). These complex endpoints still suffer in 2014 from a non-availability of alternative test methods and testing approaches accepted by the international regulatory community (*European Commission, 2013b*), as outlined in *Figure 2.1*.

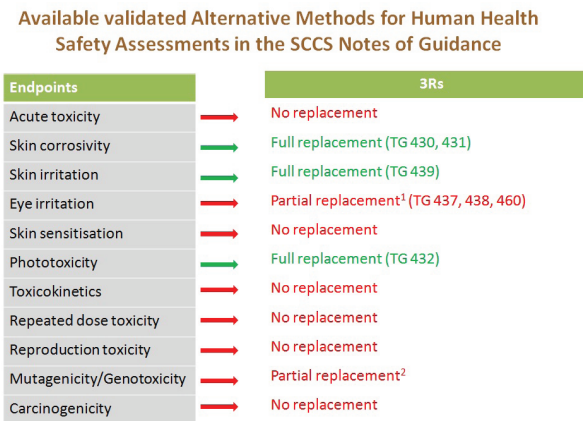

Figure 2.1 *Overview of available alternative methods for human health safety assessment – based on SCCS notes of guidance (8th revision 2012).*

It should be pointed out that the Cosmetics industry is allowed to utilise data from animal testing that have been carried out before the respective implementation dates of the marketing ban (11 March 2009/11 March 2013) to be relied on in the safety assessment of cosmetic products. Some innovation is still possible but is mostly based on use of existing ingredients. Industry is now depending more than ever on progress made in research on alternatives – the ultimate aim of the research are robust integrated safety assessment systems which relies on a rational combination of information derived from predictive tools that support a decision on a stated protection goal.

2.2.3 Cosmetics Europe's Research and Science Programme

Cosmetics industry will continue to co-fund the **SEURAT-1** Research Initiative (2011-15) together with the European Commission operating under the Health Programme of the EU's 7th Framework Programme. In addition, Cosmetics Europe has been driving its own research programme in areas of genotoxicity, eye irritation, skin sensitization, skin bioavailability and systemic toxicity and achieved considerable progress with new methods and regulatory acceptance of alternative approaches.

A new Cosmetics Europe Long Range Science Strategy (LRSS) Research Programme is anticipated to be established for 2016-20 focusing mainly on repeat dose toxicity, bioavailability (ADME) and systemic toxicity. However, designing a research programme is not just about investing in leading edge science but also about the application of the new technologies and the acceptance among regulators of using these for risk management decisions. This continued research should be underpinned by the importance of risk assessments for cosmetic ingredients based on knowledge of the toxicological properties as opposed to testing of finished products, which often does not provide meaningful data with regards to the safety of the ingredients and product. Consumer exposure to the cosmetic ingredients (including levels of systemic exposure) should also be a main part of the research with two key points to be considered; is testing definitely required for a particular toxicological end point, and if so will a non-animal test method provide usable dose response information to allow a risk assessment to be conducted? Much work is needed to develop replacement methods and Integrated Testing Strategies (ITS) for the remaining endpoints where a more fundamental understanding of the underlying biological processes and mechanisms needs to be identified before alternatives can be developed and used for regulatory purposes. However, the expectancy for when these endpoints can be covered by replacement alternatives is probably beyond a time period of 10 years; with the exemption of skin sensitisation where replacement approaches can be anticipated to come into regulatory use within the coming 2-4 years (*Adler et al., 2011*).

It is therefore worthwhile to consider trends in risk science with the specific goal of making risk assessments faster, less expensive, and more scientifically robust (*Cote et al., 2012*). In particular chemical risk assessment faces a number of challenges, including the lack of data on complex endpoints for many chemicals, the current movement away from *in vivo* toxicity testing (in extremis the EU Cosmetics regulation) and the prospect of maintaining large databases of corresponding *in vivo* and *in vitro* toxicity test results, as from *e.g.* the COSMOS project (see section 4.6). These challenges overlap with a societal need to increase the quality and utility of risk assessment information in order to provide a solid evidence base that will permit choosing among regulatory and other risk management options available to decision makers.

The way forward for the cosmetics industry's research strategy is to focus on new risk

assessment methodologies in particular on *in vitro* and *in silico* evidence to enable an improved understanding of toxicity pathways defined as "*normal cellular response pathways that are expected to result in adverse health effects when sufficiently perturbed*" (*NRC, 2007*) – all this within the classical risk assessment paradigm.

Also, the industry is increasingly asked to address broader public health and environmental health questions involving multiple and aggregated exposures, complex mixtures, and vulnerability of exposed populations – issues that some stakeholder groups often consider to be inadequately captured by current risk assessments. Because of the complexity of considering so many factors simultaneously, there is therefore a need for simplified risk assessment tools (including databases, software packages, and other modeling resources) to support a screening level risk assessment which is accessible and usable by stakeholders.

While we are striving for simplification in risk assessment procedures we should not ignore the issues we have with the usefulness of *in vitro* toxicity data for risk assessment as it highly depends on relevance of the *in vitro* data to the *in vivo* context. *In vitro-in vivo* extrapolations are necessary to understand the relevance of the compound concentration and its toxicokinetic and toxicodynamic (TK/TD) properties in both systems.

The use of the threshold of toxicological concern (TTC; section 2.3) and other exposure-based waiving approaches (such as low bioavailability) which determine the levels of substances below which there is low probability of an adverse effect should be promoted (*Dewhurst & Renwick, 2013*). The calculation of this level is based on knowledge of the chemical structure and properties of the cosmetic ingredient as well as the toxicity profiles of chemically similar substances. This level is used along with the consumer exposure scenario to the chemical to provide a risk assessment regarding the substance's safe use in a cosmetic product.

Building on current research projects and anticipated outcome the focus of the Cosmetics Europe's research in the coming years will therefore be on the following topics.

Eye Irritation

The Cosmetics Europe research programme on eye irritation has been highly prioritised for several years and provided a range of test methods and alternative approaches, including several methods validated together with the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM). It has been focused on the development, optimisation and evaluation of *in vitro* test methods for eye irritation that can be used alone or in combination in testing strategies for evaluation of cosmetic ingredients. Fundamental to this approach is identification of new or improved *in vitro* tests that use understanding of mechanisms of eye injury/recovery to better predict human ocular responses to chemical exposure. A number of alternative test methods and testing approaches have been accepted for regulatory use by SCCS and OECD and Cosmetics Europe will continue to work on and support further regulatory acceptance of alternative approaches.

Genetic Toxicity

The Cosmetics Europe Genotoxicity Task Force have been aiming at improving the specificity (reduce false positives) of *in vitro* genotoxicity tests and to develop new assays with higher relevance and predictive capacity for the dermal route of exposure. One of its activities has been to analyse key causes for the differences observed between *in vitro* and *in vivo* genotoxicity testing and is carrying out experimental work to address this with an aim to enable the use of *in vitro* assays in follow-up testing, instead of the presently required definite *in vivo* tests.

For cosmetics the most likely exposure is via the skin. Therefore the second focus area of the Task Force is the development of genotoxicity assays on the basis of 3-dimensional human skin equivalents. Using 3D skin equivalents will allow a more realistic hazard/risk assessment as they take into account the bioavailability of the substance in question as well as its metabolic fate. The strategy has been successfully submitted to SCCS and a two-test approach has been accepted for inclusion into the Notes of Guidance (*SCCS, 2014*).

Sensitization

Skin sensitization is a highly prioritised field in which a whole array of formally validated methods and ITS is expected to emerge already in the near future and Cosmetics Europe has invested considerable resources into this research area and expect regulatory acceptance by SCCS for alternative safety assessments including potency estimations within the coming 3–4 years.

The underlying mechanisms for induction of skin sensitisation is rather complex but relatively well understood and involves a number of key steps that have been described in an OECD Adverse Outcome Pathway (AOP) model (*OECD, 2013*). Virtually all key events of the skin sensitization AOP are more or less covered by *in vitro* assays, However, at present it is not possible to predict which combinations of tests into an ITS may be required to derive potency information for individual chemicals and exposure scenarios. Consequently, a tool box approach covering a majority of the AOP mechanistic steps is under review by Cosmetics Europe.

The evaluation encompasses a large set of human and Local Lymph Node (LLNA) (*Basketter et al., 2014*) data on positive control compounds and since the mechanisms of skin sensitization are fairly well understood and the individual steps are accessible to modeling. In addition, several *in vitro* models, that mimic each single step, are already available. Many of those methods have been pre-validated, and validation is ongoing for several of them. Cosmetics Europe will take an active lead in formulating ITS for the purpose of complete replacement of the skin sensitization endpoint including potency estimation and will have a first set of robust data for a number of assays for initial biostatistical analysis available by 2015.

Systemic Toxicity

Development of test methods that can be employed for systemic toxicity applications is high on the agenda for Cosmetics Europe which is clearly illustrated by the co-funding of the **SEURAT-1** Research Initiative. Cosmetics Europe will continue to work on alternatives to systemic toxicity and a considerable part of the Long Range Science Strategy (LRSS) Research Programme budget will be allocated to this endpoint.

The report published by the U.S. National Research Council in 2007 (*NRC, 2007*) changed the mindset of many scientists in relation to future toxicology testing. The concept that most late effects of chemicals can be predicted from molecular early changes (i.e. Molecular Initial Events – MIE) they cause for cellular signaling and regulation is key in designing modern toxicological assessment approaches. To support a shift in mindset in the regulatory community and to advance the science behind it, it appears logic to further build on the AOP methodology which is a well accepted approach in creating a mechanism based framework that can be used in the building of ITS or Integrated Approaches to Testing and Assessment (IATA). OECD has already in 2013 provided guidance on how to organise such information (*OECD, 2013*). It is important to note that the AOP concept frames the existing knowledge and understanding of underlying mechanisms of a clearly defined endpoint and enable the building of ITS or IATA for specific regulatory purposes. The integrated approaches may consider the entire AOP or parts of the AOP and will constitute the regulatory tool that can be used for safety assessments.

AOPs are typically represented sequentially, moving from one key event to another, as compensatory mechanisms and feedback loops are overcome. An ITS for an AOP is often applied following a so-called 'bottom-up approach', where chemistry and mechanistic information are initially used in the process of hazard identification. An ITS approach can also be used in a 'top-down approach', by taking the final adverse outcomes produced by well studied substances and establishing modes-of-action (MoA), then using information to e.g. develop chemical categories such as in the International Programme on Chemical Safety (IPCS) conceptual framework for evaluating a mode-of-action (*Sonich-Mullin et al., 2001*).

Whilst AOPs may be depicted with a single axis, toxicity is multi-dimensional (e.g. gender, species), so the pathway between a MIE and the final adverse effect can vary significantly. This is especially true for more 'complex', longer-term endpoints, where effects are the result of multiple organ interactions (e.g. skin sensitisation), multiple events (e.g. repeated dose toxicity), accumulation over time (e.g. neural toxicity), or are related to a specific life stage of an organism (e.g. developmental toxicity). Nonetheless, although a number of biochemical steps are required for a toxic response to be realised, the MIEs are a prerequisite for all subsequent steps *(Enoch and Cronin, 2010)*.

With that said, it is understood that a single MIE may impact several signaling cascades and, based on current knowledge, these signaling cascades may cause opposing events.

Additionally, an AOP is based on the fact that chemical interactions are at the molecular level and not at the whole organism level. Thus, adverse effects observed *in vivo* are the result of biological cascade initiated by the chemical structure of the toxicant.

There is growing appreciation that the conception, design and execution of experimental investigations to explore toxicological mechanisms in support of AOP development is a tedious undertaking which requires considerable resources and high level of expertise. Cosmetics Europe is acknowledging this and is at the same time cognisant that mining available databases reporting both *in vitro* and /or *in vivo* studies (toxicodynamics and toxicokinetics) can provide an invaluable source of MoA information to support AOP development. In addition, proof of concept studies (i.e. predictive toxicity case studies) appear particularly important for assessing whether the new approaches are feasible based on the current stage of knowledge. It would be far too ambitious for one organisation or sector to force progress in this area – it requires a collaborative effort with defined goals and objectives – a challenge for a new research effort in 2016-2020 by Cosmetics Europe.

Systemic Bioavailability / ADME

Cosmetic ingredients are per definition low toxicity chemicals due to their intended applications to the skin, hair or the mouth. Determining the systemic bioavailability of an ingredient is important for the further evaluation of potential systemic toxicity and constitute an area of research of central interest to Cosmetics Europe. The extent to which a substance is systemically available is an important parameter for determining nominal/real concentration levels and therefore essential for *in vitro* to *in vivo* extrapolations. Toxicokinetics (TK) studies factors influencing the time and concentration course for absorption, distribution, metabolism and excretion (ADME) of potential or actual toxicants (chemicals) within the body. In physiologically-based toxicokinetic (PBTK) modelling, the compartments which the chemical can reside in or distribute between correspond to physiological tissues with appropriate volumes, blood flows, and pathways for metabolism of compounds. PBTK models are founded on methods established in the pharmaceutical sector for predicting kinetic profiles of drug-like chemicals in blood and tissues following administration to humans.

For pharmaceutical purposes characterising the ADME properties for most chemicals requires the use of *in vitro* assays or *in silico* estimation using quantitative structure activity relationships (QSAR). In particular linking the bioactivity or pathway-perturbations observed in the *in vitro* models at specific nominal concentrations to concentrations of the chemical in the target tissue or blood *in vivo*. For that, research is required to apply PBTK modelling in conjunction with targeted *in vitro* biokinetic studies to quantitatively translate *in vitro* observations into equivalent *in vivo* dose metrics.

Currently, there are a number of *in vitro* and *in silico* methods to cover different aspect of the toxicokinetics processes. Although for some of them a further development/improvement is still necessary, some others of the existing methods are already well developed, but, their relevance and reproducibility needs to be established. Cosmetics Europe foresees considerable research needs in this area with an aim towards providing conceptual case studies for safety assessments of cosmetic ingredients that can be used to further build on for specific scenarios of regulatory acceptance.

2.2.4 Achieving Regulatory Acceptance

Validation is a controlled process for achieving the regulatory acceptance of a test method or approach by demonstrating the relevance and reproducibility of a test to ensure its safe use in risk assessment. Traditionally validation follows the criteria of OECD Guidance Document no. 34 (*OECD, 2005*) and constitutes a considerable investment both in terms of resources and time. Considering that new assessment tools for regulators will comprise more and more combined strategies of mechanistically relevant *in vitro* assays the focus of future validation efforts should be more on the reproducibility of the test methods, e.g. in high throughput screening systems (HTS). Considering the needs of the Cosmetics industry to develop replacement methods by combinations of *in vitro* systems and the large numbers of *in vitro* tests that will become available in the coming years, it is expected and warranted that the present validation procedures are reconsidered and made fit for purpose. The validation and regulatory acceptance of relatively straight forward *in vitro* tests may take a decade to conclude and the considerable resource implications for test method developers points at the urgency of establishing a streamlined and flexible validation model providing faster and cheaper validations, yet providing a high level of safety for the consumer. Considering the expected increased use of AOP-based ITS/IATA and incorporation of HTS information, novel validation criteria and novel test method standards should be incorporated into the OECD Test Guideline/AOP concept and here EURL ECVAM and OECD will play a crucial role for success. It could be envisaged that work in an expert consensus procedure (e.g. governed by SCCS) to set up standard operating procedures by consensus could be promoted and validation for a defined purpose could be performed in testing the reliability of the methods with compounds possessing properties relevant for cosmetics.

The on-going paradigm shift towards mechanism based approaches such as AOP is well established and several major governmental organisations in e.g. USA and EU are investing heavily into this new area of risk assessment tools. Cosmetics Europe welcomes this development toward deeper understandings of mechanisms in our *in vitro* tests and acknowledges the important international efforts by the OECD to create new global standards in the area. The commitment by the OECD also enhances the understanding of the concept by the regulatory community and will enable new pragmatic uses of AOP based approaches.

The skin sensitization AOP is a fine example of how international agreements give a framework for further research and test method development and Cosmetics Europe is adapting its work in the skin sensitization area fully towards compliance with the OECD AOP. Nevertheless, it is not anticipated that there will be AOPs available in the short to medium term for the more complex endpoints and international collaborative efforts to outline suitable endpoints for AOP development is fully supported by Cosmetics Europe.

Cosmetics Europe will continue to work with its research partners to take advantage of new scientific insights and technologies to improve its toolbox for assessing substances for safe use in Cosmetic products and a new Long Range Science Strategy (LRSS) Research Programme for 2016-20 is under establishment, a timeline for this effort is illustrated in the schedule below (*Figure 2.2*). In the past, Cosmetics Europe has worked together with the research/science community in various ways but always in a fit for purpose manner – the new LRSS programme will be conducted and managed in a way that takes advantage of this experience and the outcomes from the **SEURAT-1** Research Initiative and the existing research programme within Cosmetics Europe. There will be no restrictions on how the Cosmetics Europe research programme in terms of leverage and collaborations will be executed what matters is the industry's need to support and conduct the best science with highest relevance and applicability for the industry. And to achieve this Cosmetic Europe will establish working relationship with relevant partners both within the EU as well as internationally.

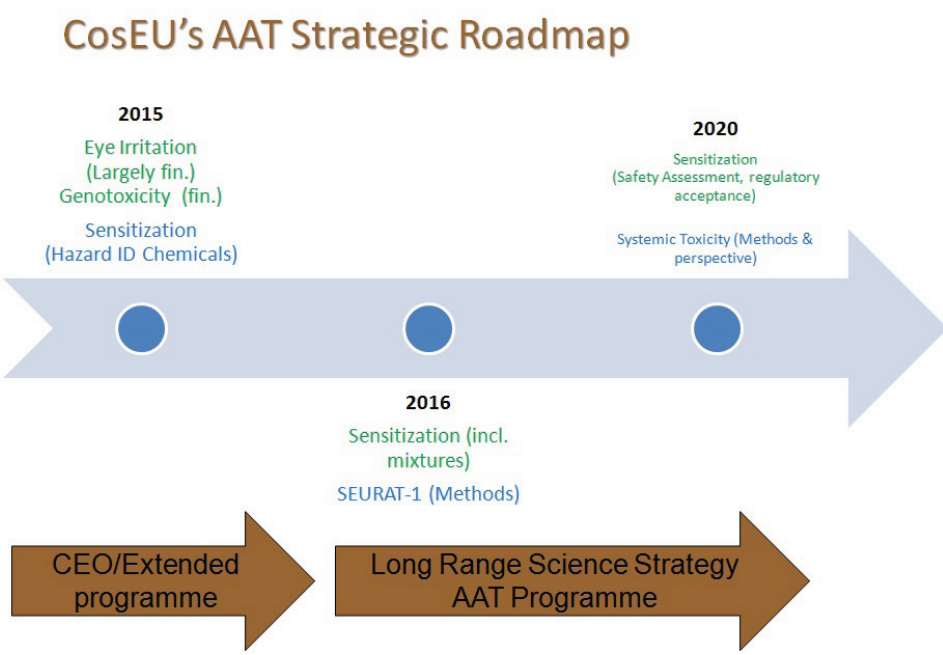

Figure 2.2 *Strategic roadmap for Cosmetics Europe AAT research programme.*

Innovation drives the cosmetics industry to provide new, safe products for the millions of people who use them every day, throughout their lives. The industry's capacity to innovate is fundamental to meeting consumers' constantly growing expectations and the new Long Range Science Strategy (LRSS) Research Programme will continue to ensure the safe use of cosmetic products in the EU and globally.

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2.3 The Concept of Threshold of Toxicological Concern and Recent Trends

Chihae Yang, Mitchell A. Cheeseman, Andrew Worth

2.3.1 Introduction

From the perspective of national and international regulatory policies such as Canadian Domestic Substances List (DSL), European Union's REACH and Cosmetics regulations, and ICH M7 for pharmaceutical impurities, the need to implement efficient evaluations of chemical safety for human risk assessment has become increasingly important. Any non-testing method should be pragmatic and transparent with clear constraints. One of the non-testing methods that allows a preliminary risk assessment of chemicals based on the availability of reliable exposure information is the Threshold of Toxicological Concern (TTC). Thus, there

is increased interest in broadening the use of the TTC concept within the regulatory context. Currently the TTC concept continues to be used by the international regulatory community, most notably for the evaluation of flavouring agents by the FAO/WHO Joint Expert Committee on Food Additives. It has also gained acceptance for the evaluation of genotoxic impurities in pharmaceuticals and natural health products. The following historical overview describes the current status of TTC, which leads us to the TTC of the 21st century in the light of the new toxicological paradigm.

2.3.2 Historical Insight

The concept of Threshold of Toxicological Concern was initially developed from efforts by the Center for Food Safety and Applied Nutrition (CFSAN) of the US Food and Drug Administration (FDA) to address the challenges in the safety assessment of food contact substances. The regulation of food contact substances, involving very low-level potential exposure to a wide range of chemicals migrating to food, posed significant challenges regarding regulatory and testing efficiency. In 1967 Frawley first proposed a threshold based on his analysis of chronic toxicity data for 220 chemicals, which would result in no hazard to health of the consumers of foods because of the low level of exposure involved in food packaging materials (*Frawley, 1967*).

Two decades later as more comprehensive toxicity databases began evolving, *Rulis* (*1986; 1989*) published an analysis of classical toxicity and carcinogenicity data in support of a threshold of regulation. *Rulis* compared LD_{50} values for compounds in FDA's Prioritized Assessment of Food Additives (PAFA) database (*Benz & Irausque, 1991*) and the Carcinogenicity Potency Database (CPDB; http://toxnet.nlm.nih.gov/cpdb/). He also compared LD_{50} values with lowest effect levels (LELs) for compounds in PAFA and with the risk equivalent doses (one in one million) of carcinogens from the CPDB. This comparison demonstrated the substantial margin between those dosing levels that produce toxic effects and the typical exposure to substances used as contact substances. The margin of exposure suggested that carcinogenicity was the endpoint of most concern at the lowest dietary concentrations, presumably in large part due to the extrapolation to low risk levels on the order of one in one million as compared to traditional safety factors.

These two decades of research activities led FDA to adopt the 'Threshold of Regulation (TOR)' to exempt from the requirement of a food additive listing regulation any substance used in food-contact substances (e.g., food-packaging or food-processing equipment) that migrates, or that may be expected to migrate, into food, if it becomes a component of food only at levels that are below the threshold of regulation (see Title 21 of the U.S. Code of Federal Regulations (CFR) section 170.39; *FDA, 1995*). Specifically, an identified migrant of known chemical structure can be exempted if the incremental dietary concentration is below 0.5 μg/kg of diet and the substance has not been shown to be a carcinogen in humans or

animals. If the FDA is satisfied that the conditions for exemption are met, the chemical does not ordinarily have to undergo toxicological testing nor the formal pre-market safety evaluation by the agency. The dietary concentration of 0.5 μg/kg-diet is equivalent to an intake of 1.5 μg/person/day or 25 ng/kg-bw/day based on an adult of 60 kg-bw with 1500 g each of food and liquid intake. The TOR is intended to be protective for all toxicological endpoints, including carcinogenicity, although U.S. law does not permit known carcinogens to be regulated as food and colour additives according the Delaney Clause (http://www.fda.gov/AboutFDA/WhatWeDo/History/Overviews/ucm056044.htm).

The premise of the TOR was further supported by *Cheeseman et al.* (*1999*). This publication confirmed that carcinogenicity is the endpoint of most concern at the lowest dietary concentrations by comparing one in a million risk level with the toxic effects at the lowest dose reported for chemicals in the RTECS (Registry of Toxic Effects of Chemical Substances) database; http://accelrys.com/products/databases/bioactivity/rtecs.html). The authors derived the 'pseudo-acceptable daily intake (PADI)' by analysing the TDLo (toxic dose low) values from the reproductive and developmental toxicity studies (> 3,000 test substances) and multi-dose experiments (> 2,500). They then compared the PADIs from the threshold values based on the TD_{50} distribution of 709 substances selected from the CPDB database. The significance of this study is that these results support that a 'virtually safe dose (VSD)' based on rodent carcinogenicity data would also protect against other toxic effects (*Cheeseman et al., 1999: Barlow, 2005*). In the same study, the authors also reported an important finding that structures containing genotoxic alerts tend to be more potent carcinogens than structures that do not. FDA's implementation of its TOR review process saved the agency an estimated 100-150 staff years in review time over its first 10 years of implementation while permitting closer regulatory control over low-level exposures to food contact substances (*Cheeseman, 2014*). *Barlow* also reported in 2005 that in the first 10 years since its implementation, the TOR has generated significant efficiencies for FDA (*Barlow, 2005*).

Although the origins of the cancer TTC concept are thus in regulation of food contact substances at US FDA, much attention had already been given for the extension of the concept to non-cancer endpoints during the same decade. In 1996, *Munro et al.* (*1996*) proposed a correlation of structural classes with NOEL (No Observed Effect Level) values in an attempt to establish non-cancer thresholds based on 613 test substances with repeated-dose toxicity data from sub-chronic (38%), chronic (33%), and reproductive/developmental (29%) studies. For structural classes, the authors employed the Cramer decision tree method (*Cramer et al., 1978*) to classify substances into three groups of oral toxicity potential, i.e., low toxicity to Class I (137), moderate toxicity to Class II (28), and severe toxicity to Class III (448). The Cramer analysis was one of the first to report the association of structural groups to potency based on analysis of a large number of diverse chemicals including pesticides, cosmetics, food additives, drugs, and industrial and environmental chemicals with known biological properties. The fifth percentile NOEL of each Class was then derived from the distribution and divided

by a safety assessment factor to derive corresponding human exposure thresholds. To this date, the resultant human exposure thresholds of 1.8, 0.54, 0.09 mg/person/day for Cramer Classes I, II, and III respectively, are still being used.

The Cramer decision tree, devised in 1978, basically follows 33 logical rules to classify chemicals according to various structural classes based on associated toxicity and metabolism knowledge. It is worth mentioning that the interpretation of these expert rules can become complicated, leading to the potential for conflicting classifications. The 1978 publication presented an analysis of NEL (no effect level) values from 88 compounds in relation to the development of the Cramer decision tree. The separation of log(NEL) distributions for the three Classes was demonstrated. As noted in *Table 2.1*, four orders of magnitude separation of the NEL between Cramer Classes I and III was clearly demonstrated based on 80 compounds. When the database was expanded by Munro in 1996, such a large separation was no longer observed although there were sufficiently large (>3 fold) differences in the NOEL (no observed effect level).

Table 2.1 *Comparison of potency ranges of Cramer Classes.*

	Cramer 1978		Munro 1996	
	NEL (mg/kg/day)	N	(NOEL mg/kg/day)	N
Class I	50 – 254	31	0.018 – 7204	137
Class II	5 – 200	7	1 – 1441	28
Class III	0.03 – 500	50	0.005 – 3775	448

The authors mentioned that the procedure had been applied to a large number of pesticides, drugs, food additives, and industrial and environmental chemicals, although the method was originally developed for the safety evaluation of food flavouring substances. Although Munro's 1996 dataset indeed validates this statement, it is probably fair to point out that we cannot be so certain that these Cramer Classes will still be upheld as we include more knowledge on chemical spaces not well represented in *Munro et al.* (*1996*).

During this period, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) was tasked with evaluating more than 2,500 flavouring substances where only representative toxicity or metabolic data existed for each chemical class of flavours. Upon adopting Munro's proposed method (*Munro et al., 1999*), JECFA was able to facilitate the evaluation; for example, the evaluation of over 1,400 flavouring agents within this period was possible compared to that of only 70 agents before 1996 (*Barlow, 2005; Larsen, 2006*). The major modification made by JECFA was to incorporate expert judgment to determine whether the approach can

be extended to metabolites of flavouring substances. The test substance is not expected to be of concern and specific toxicity data are not required if the estimated daily intake is below the TTC value for the respective Cramer Class or the substance is a flavouring chemical or its endogenous metabolites. In the case of unidentified constituents, the Committee recognised that a general threshold could not provide the same reassurance of safety as in the case of structurally defined compounds. However, it was agreed that the value of 1.5 µg/person/day for unidentified components of flavour complexes derived from natural sources could be incorporated into a pragmatic approach for establishing analytical requirements (*Munro et al., 1999*). The European Commission's Scientific Committee for Food (SCF) evaluated the JECFA procedure (*WHO, 1997; 1999*) and in 1999 recommended to adopt the TTC approach, with some modification, for use in the evaluation of flavouring substances in the European Union (*European Commission, 1999*).

Combining the results from the development of both cancer and non-cancer TTC approaches, a decision tree was proposed by *Kroes et al.* (*2004*) to be used as guidance on when and how the TTC could be applied in food safety evaluation. *Figure 2.3* illustrates the TTC decision tree derived by the authors (ILSI Europe TTC expert group) for assessing substances without further compound-specific testing. At the start of the process, the compound is checked to determine whether it belongs to one of the chemical classes for which the TTC approach is not appropriate. (Q: Is the substance a non-essential metal or metal-containing compound, or is it a polyhalogenated-dibenzodioxin, -dibenzofuran, or -biphenyl?) The compound can also be screened for genotoxic alerts before being allowed to proceed to apply the threshold for cancer. If the chemical is matched with a lower concern genotoxic structural alert (i.e., not containing azo/azoxy or N-nitroso moieties or a aflatoxin-like structure) the cancer threshold (0.15 µg /day) is compared with the exposure of the chemical. The non-cancer portion of the decision tree begins with a question whether the chemical is an organophosphate to which a lower class specific exposure threshold can be applied. The non-organophosphate chemical is allowed to proceed to the node for the comparison of the exposure with the threshold of the appropriate Cramer Class. If the exposure exceeds the threshold, the risk assessment requires compound-specific toxicity data.

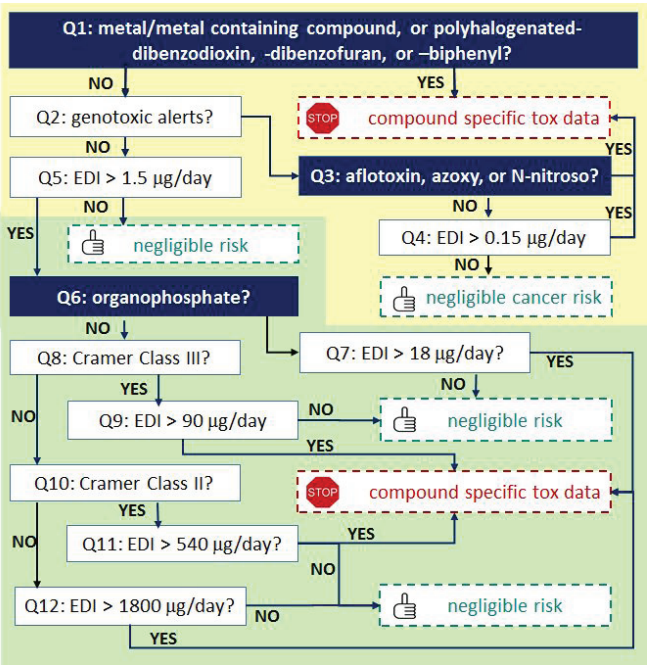

Figure 2.3 *TTC Decision Tree for combined cancer and non-cancer endpoints.*

2.3.3 Current State-of-the-Art

Extending the present applicability domain of the TTC approach to substances other than food additives, contact substances, fragrances, and pesticides and their metabolites is being actively pursued internationally. Two important factors beyond the availability of reliable toxicity data include the chemical applicability domain of the database from which the thresholds are derived and the bioavailability issues involved in the exposure scenarios. The applicability of the Munro 1996 dataset to other chemical domains such as cosmetics, antimicrobials, or air pollutants has been considered by several initiatives (*Blackburn et al., 2005; Escher et al., 2010; Pinalli et al., 2011; Kalkhof et al., 2012*). As shown in *Figure 2.4*, the chemical space of the Munro dataset is quite diverse, broadly covering a variety of substances types including industrial chemicals (396), agrochemicals (207), cosmetics-related substances (189), food additives (147), and pharmaceuticals (147). It is also worth noting the high number of both agrochemicals and cosmetics-related substances in this dataset. Other than the intentional exclusion of steroids, inorganics and organometallic compounds, the Munro dataset is remarkably well-balanced. However, coverage is poorer for some chemical classes important for cosmetics or antimicrobials, including hair dyes, non-ionic and cationic surfactants, and cyanuric rings. Overall, its chemical diversity is one reason why Munro is still being used directly or by comparison to new chemical applicability domains.

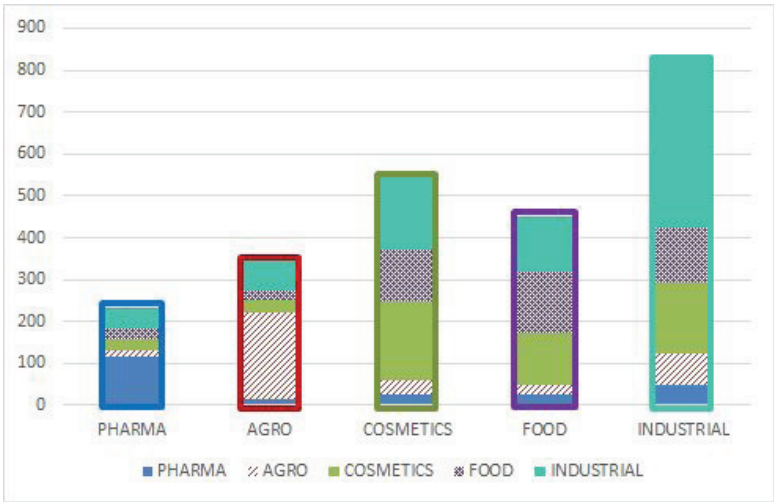

Figure 2.4 *Substance use types of Munro TTC dataset.*

As the TTC approach has been considered for application to chemical exposures other than through the food supply, the fact that the underlying data is from oral toxicity testing has become a critical issue. In applying the TTC concept to other chemical applicability domains where the route of exposure is not oral, two approaches are possible to address the bioavailability differences. One is to build a new toxicity database comprised of studies conducted with the appropriate route of exposure. The other would be to use the oral TTC values as the basis for an oral-to-non oral extrapolation, as is often done in conventional risk assessment in cases where only oral toxicity data are available. *Kroes et al.* (*2007*) concluded that conservative default adjustment factors could take into account the relationship between the external topical dose and the internal dose, followed by application of the oral TTC values derived by *Munro et al.* (*1996*). However, route-specific metabolism was also identified as a critical consideration. The authors also noted that the TTC approach relates to systemic effects and may not be protective of local effects at the site of application. Currently two TTC initiatives have been working to address the oral-to-dermal extrapolation for cosmetics-related chemicals: ILSI Europe COSMOS TTC (*Williams et al., 2014a; 2014b*) and antimicrobials TTC by ILSI Research Foundation (*Guy et al., 2014*). Both working groups have found that it is critically important to understand bioavailability, skin permeability, metabolism, and biokinetic (PK/TK) profiles.

Current applications of the TTC approach include food contact materials, genotoxic impurities in pharmaceuticals, metabolites of plant protection products in groundwater, flavouring substances, and food contact materials. Potential applications are being considered for foods, food additives, contaminants, medical devices, residues from veterinary medicinal products,

industrial chemicals, air pollutants, and cosmetics and other consumer products. In 2012, both the European Food Safety Authority (EFSA) and the Scientific Committees of the European Commission published opinions on the applicability of the TTC approach (*SCCS/SCHER/SCENIHR, 2012; Aungst et al., 2012*).

The EFSA opinion included an evaluation of the relevance, reliability and applicability of the TTC approach as a tool for providing scientific advice about possible human health risks from low-level exposures. Further, also discussed were additional areas within EFSA's mandate where TTC is potentially of value. A role for tiered approaches to toxicological testing was also envisaged, where data requirements are linked to the level of human exposure. Limitations to this approach were also described in this report.

Although some studies note that Cramer Classes may overestimate hazard (*Kalkhof et al., 2012*), EFSA found them still conservatively protective. Application of the 100 fold uncertainty factor to the 5th percentile NOEL results in a TTC value that is approximately 17-fold and 3-fold lower than the lowest NOEL values of Cramer I and III classes, respectively, in *Munro et al.* (*1996*) dataset. Thus, the EFSA opinion confirmed that the lowest NOEL value in the distribution is covered. However, EFSA noted that the TTC value for Cramer Class II is not well supported by currently available databases. The TTC value for Cramer Class II substances derived by *Munro et al.* (*1996*) was based on toxicological data on very few substances. Databases compiled subsequently have similarly found few chemicals classifiable as Cramer Class II, apart from flavouring substances. Therefore EFSA concluded that consideration should be given to treating substances that would be classified in Cramer Class II under the Cramer decision tree as if they were Cramer Class III substances.

In 2012, the three independent non-food Scientific Committees of the European Commission – the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) – were jointly tasked with the evaluation of potential applications of the TTC approach for human health risk assessment of chemical substances, with particular focus on cosmetics and other consumer products (*SCCS/SCHER/SCENIHR, 2012*). The Scientific Committees published a full review on the various aspects of the TTC approach, including classes of chemicals, exposure situations, and toxicity endpoints which may be addressed using the TTC concept, as well as the quantity and type of information (exposure, toxicity, QSAR, statistics, etc.) required for a particular class of chemicals and/or exposure situation before the TTC concept can be applied in risk assessment.

The Scientific Committees considered the TTC approach, in general, 'scientifically acceptable for human health risk assessment of systemic toxic effects caused by chemicals present at very low levels, as based on sound exposure information.' As in the *EFSA* (*2012*) opinion, they noted that the TTC is a probabilistic screening tool (with uncertainty) and that TTC values are not based on the lowest value in each of the frequency distributions but on a point close to the

lowest value. Thus, the possibility exists that a substance with an exposure below the relevant TTC value may still pose a potential risk for consumer health or a lifetime cancer risk greater than one in a million. In this regard the 2012 analysis by EFSA described above [28] provides a positive insight by noting that the non-cancer TTC values are derived from no effect levels.

For substances with genotoxicity alerts, the Scientific Committees considered the current default value of 0.15 µg/person/day adequate with a recommendation to strengthen the scientific basis. For substances with no structural alerts for genotoxicity, the division into Cramer Classes I and III was found to be acceptable in principle. However, as in the *EFSA* (*2012*) opinion, the TTC value of Cramer Class II was determined not to be adequately supported by the available databases and was suggested that Cramer Class II substances be treated as Class III. The workflow depicted in *Figure 2.3* would still be valid except for the handling of Class II.

The Scientific Committees emphasised the need for expanding the applicability domain of the database, and the importance of having a high level of confidence in the quality and completeness of the toxicity data, the reliability of the exposure data for the intended use of the chemical, and the appropriateness of any extrapolations (e.g., route-to-route) in order to apply the TTC approach in risk assessment. Therefore, they considered that the TTC approach should be applied on a case-by-case basis and requires expert judgment and in-depth knowledge in both toxicology and exposure assessment. In relation to cosmetics, the Committees indicated the current databases require further development and validation, and the TTC concept can only be applied to those cosmetic ingredients which belong to a sufficiently well-represented structural class in the TTC database and where appropriate exposure data are available.

Both the EFSA and the EC Scientific Committees agreed that the use of TTC approach should not be applied to the following categories of substances, since they are either known to be classes of high human health concern or are not (adequately) represented in the underlying TTC dataset: highly potent carcinogens (i.e. aflatoxin-like, azoxy-, N-nitroso, benzidine, hydrazine), inorganic substances, metals and organometallics, proteins, steroids, bioaccumulating substances (either known or predicted), nano materials, radioactive substances, mixtures of substances containing unknown chemical structures.

2.3.4 Threshold of Toxicological Concern in the 21st Century

As described at the beginning of this section, the TTC was a pragmatic approach related to FDA's TOR policy. Cases have been presented where implementation of the TTC approach greatly increased productivity. At the heart of this approach, two components are central: the toxicity data (with a clear method to determine the point of departure) and the chemistry tool used to classify chemicals into potency classes.

The first component is the toxicity database. The underlying databases used to derive the thresholds that are still used have been scrutinised in recent years. Some of the Munro data have been reviewed by EFSA and the ILSI Europe COSMOS TTC project. US FDA has also reviewed the study inclusion criteria of the CPDB database used for cancer TTC (*Aungst et al., 2012*). New toxicity databases are being made publicly available with the studies conducted after publication of the *Munro* and *Cheeseman* papers. To name a few, the ToxRefDB (http://www.epa.gov/ncct/toxrefdb/), RepDose (http://www.fraunhofer-repdose.de/), oRepeatTox DB within COSMOS DB (http://cosmosdb.cosmostox.eu), and HESS (http://www.safe.nite.go.jp/english/kasinn/qsar/hess-e.html) databases are now available. The data quality can be assessed by data record accuracy and results acceptability. The data record accuracy has been improved dramatically due to modern technology; however, results acceptability using these databases does not necessarily parallel data record availability. With the new regulatory science paradigm, the vast amount of screening data generated from Tox21/ToxCast™ (http://www.epa.gov/ncct/Tox21/) are still being evaluated for how to best apply such information within the regulatory setting. The AOP (adverse outcome pathway) concept promises the elucidation of molecular pathways connecting the molecular initiating event to the final outcome through a series of events in cellular/organ/organism. As promising as this concept appears to be, it remains to be seen when and how the necessary knowledge of mechanistic reasoning can be provided from the vantage of toxicity data.

For the second component of the TTC, the Cramer Decision Tree is currently used as the chemistry tool. As pointed out in the previous section, these rules are derived from underlying toxicity data available in 1970's. Whilst good toxicity studies are still valuable regardless of when they were conducted, testing protocols have changed and our mechanistic understanding has improved and expanded during the past 40 years. There has been emerging needs to group compounds with categories that are more refined than just Cramer Classes, especially, when the mode-of-action is well-understood. To this end, there are two ways to go about modifying the current approach. One is to adapt the TTC decision tree by devising additional nodes. For example, using a separate class of 'organophosphates' was necessary in the current TTC decision tree since the Cramer Class did not further differentiate its potency within Class III. Studies to promote the extension of such ideas to carbamates (*SCCS/SCHER/SCENIHR, 2012; Leeman et al., 2014*) and organohalides (*Muldoon-Jacobs et al., 2014*) have been also reported. These approaches support keeping the Cramer Classes, but devising many structural classes within the TTC decision tree to satisfy the need to provide a more refined structural categorisation than the Cramer classification currently allows, an enhancement that reflects mechanistic rationale. Another way to address this issue is to devise a new structural classification method based on recent toxicological knowledge and the application of modern chemistry-based tools.

The second implication relates to the new science and technology involved in the field of

chemoinformatics. The Cramer rules were developed when chemical structures had to be drawn manually by humans on paper. Validating the rules against a dataset required that a human expert assess them one at a time. Although our basic knowledge of chemistry and Structure Activity Relationships (SAR) may not have fundamentally changed since these early efforts, modern computational approaches to design substructure patterns to differentiate the compounds with different biological modes-of-action can much better be refined when the rules can be clearly represented and matched against the target structure set. This has two implications: (i) the original rules could have been designed better if modern technologies had been available at that time; (ii) given that we can now take full advantage of current technology, these rules can be applied with greater accuracy and consistency if software programs are used rather than human experts. In this context, the use of Toxtree and OECD toolbox to apply the Cramer rules to assign the classes was actually a necessary step. These two issues affect not just the technology of the TTC methodology, but the science as well. Coding rules into software demands that they have precise and logical definitions, and that there are no conflicts between different rules; a well-designed knowledgebase software system is thus more consistent and accurate than human experts. The ambiguity of rules presents a serious problem for knowledgebase systems, regardless of possible issues with the underlying chemoinformatics technology. Recently some TTC initiatives realised that in the case of some chemicals the Cramer Class assignments by Toxtree and the OECD Toolbox conflict with each other, or may not be in agreement with the conclusions of human experts. These conflicts were reported by both COSMOS TTC and RIFM groups (*Muldoon-Jacobs et al., 1014; Bhatia et al., 2014*). Considering the compromise between a set of 40-year old rules implemented on different chemoinformatics platform, these differences are not too surprising.

The Cramer rules would have been written much more clearly if the current chemoinformatics science and technology had been available. It is also true that modern chemoinformatics would not approach the design of potency-driven categories solely based on substructures or classify compound potency following Cramer-like rules. In the Antimicrobial TTC project, a set of antimicrobial chemotypes has been designed (*Cheeseman et al., 2014*). Chemotypes are defined as substructures encoded with physicochemical properties that can carry biological information (http://chemotyper.org, http://toxprint.org). These chemotypes were correlated with the potency through NOAEL/LOAEL values and used to group antimicrobials whose compound-specific toxicity data were not available. The ToxPrint chemotypes are publicly available and can be applied using public tools. In the current ChemoTyper version, the cancer potency TTC categories defined by *Kroes et al.* (*2004*) are already implemented, in addition to the ToxPrint chemotypes. To help group compounds, a similar approach is being pursued in COSMOS TTC project and some of the cosmetics chemotypes have been already published in a JRC report.

The Scientific Committees of European Commission recommended that when using the TTC

approach, any available information on the toxicity of the chemical and structurally related chemicals should still be considered, including structure-activity relationship (SAR) and read-across analyses. In this 21st century regulatory science, should we not challenge ourselves to group chemical classes according to chemotypes representing the relationship between biology and chemistry properties? In our new century, the lines between TTC, (Q)SAR, and read-across have become blurred; this is not a problem but rather an inevitable temporary state of affairs as the field continues to advance, moving forward in the right direction. In the future, we can envision the traditional TTC approach based on the 'low-resolution' and chemically diverse Cramer Classes being replaced by the use of 'high-resolution' and mechanistically meaningful groups of substances, defined by chemotypes, and enabling the filling of toxicity data gaps through the application of read-across or QSAR. The user could then decide on the appropriate choice of safety assessment factors, according to the uncertainty in the prediction (which needs to be well characterised and documented), and the decision-making scenario.

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2.4 Implementation of Non-standard Data into Safety Assessment: Dealing with Uncertainties

Derek J. Knight, Tomas Öberg

2.4.1 Introduction

There are two needs for assessing the hazardous properties of chemical substances:

- To screen a large set of substances to select groups with particular characteristics for further action.
- To assess a specific substance for a defined purpose, e.g. to fill a 'data gap' and establish safe use from a risk assessment.

The degree of uncertainty tolerated in the prediction depends on the regulatory purpose: therefore assessing and communicating uncertainty is a key element. In general, higher certainty is needed to assess specific individual substances than for screening sets of chemicals for priority setting. The WHO/IPCS mode-of-action/human relevance framework, which has been updated to reflect experience in applying it and to extend to emerging areas in new methods, gives insight into problem formulation for the regulatory purpose in driving the need for more robust information and less uncertainty *(Meek et al., 2014).*

Regulators generally set standards for the information on the properties of chemicals, whether from standard tests or non-standard approaches; hence if the prediction does not meet the standard it is not fit for purpose. For example, for registration of a chemical under the REACH Regulation, such non-standard information has to be equivalent to the information obtained from the standard studies, in that the key parameters of the standard method should be addressed and the result must be suitable for adequate risk assessment and classification. Also registrants have to justify these adaptations of the standard information requirements in the registration dossier by providing scientific explanations.

2.4.2 Non-standard Information Using Combined-Approaches

There is a wide range of properties assessment for chemicals: 'traditional' toxicology studies, *in vitro* tests, 'read-across'/'chemical categories', quantitative structure activity relationships (QSARs) and 'high throughput screening' approaches. These approaches can be combined in a weight of evidence (WoE) as a rational integration of tests data and predictions into integrated testing strategies (ITSs)/integrated assessment and testing approaches (IATAs) and/or 'batteries of tests'. The underlying biological mechanisms that underpin toxicity

should be used as the basis and to support such combined approaches. Mode-of-action (MoA) considerations and adverse outcome pathways (AOPs) facilitate thinking into how to derive combined approaches. Refinement methodologies can improve knowledge and lower uncertainty; e.g. PBPK modelling (*WHO, 2010*), Chemical Specific adjustment factors (*WHO, 2005*) and non-test methods or *in vitro* assays for *in vitro* to *in vivo* extrapolations.

Combined-approach assessment techniques should be fit for the purpose the prediction is to be used for and also flexible enough to allow substance-specific adjustments.

The **SEURAT-1** 'conceptual framework' is a higher-level method for bringing together evidence at different levels of biological organisation to predict repeated-dose toxicity, encompassing AOPs within the framework. This framework is intended to set out a structure to guide assessors in devising a fit-for-purpose 'bespoke' IATA for the particular circumstances and case. The overall outcome is anticipated to be robust as it is not based on single pieces of evidence, rather a weight of evidence combined in a biologically-rational matter (further details are given in the report of the **SEURAT-1** Safety Assessment Working Group in section 4.11.8).

2.4.3 Approaches to Deal with Uncertainties

There are different types of uncertainty to address in safety assessments and there are different suggestions for classification (taxonomy) and how to systematise this (*Morgan & Henrion, 1992; Regan et al., 2002*). An important distinction is between uncertainty due to lack of knowledge (epistemic uncertainty) and uncertainty arising from variability and inherent randomness in the systems under study (aleatoric uncertainty). When discussing empirical quantities, epistemic uncertainty is synonymous to systematic errors or bias, and can in principle be reduced or even eliminated with better knowledge. Variability, on the other hand, cannot be reduced only better characterised. All biological, chemical and technical systems display some degree of variability and a typical example of relevance for safety assessments is the variability between individuals of a population.

When implementing non-standard data into safety assessments all kinds of uncertainty need to be considered, but usually the focus is on bias. Systematic errors may for example arise from subjective judgements, inaccurate choice and structure of models or non-representative reference data. *In vitro* tests, read-across, QSARs and WoE approaches are in principle all to be seen as models similar to the animal test models to be replaced or supplemented.

A scientific approach to uncertainty requires that it is identified and assessed. A scientific assessment of an empirical quantity therefore always requires an additional assessment of the uncertainty around this quantity. Probability distributions is well suited to characterise variability factors, but less so for knowledge uncertainty (bias). The simple reason is that to assign probability distributions we need a lot of information, not only a best estimate and possible range (minimum and maximum), but also how the probability varies within this range.

A straight-forward approach is instead to assign a range, i.e. to characterise uncertainty as an interval. In risk assessment this approach is often taken, since assessment factors can be seen as one-sided assessments of an interval. A similar approach is possible and has been suggested for non-standard approaches by adding extra (higher) assessment factors (*ECHA, 2008*).

We may also have additional information, observations or expert knowledge, which justifies further characterisation of the uncertainty. It may for example be possible to give the best estimate with an interval around or maybe also to differentiate between reasonable and absolute maximum/minimum ('worst case'). Likewise, if we have justification for using a probability distribution, we still need to characterise the uncertainty in this selection and not only for the defining parameters. Both these examples indicate a need to combine approaches to describe uncertainty, for example intervals and probability distributions.

Probability bounds analysis (PBA) has been introduced as a method of investigating the full extent of uncertainty, including the selection of input distributions (*Ferson & Ginzburg, 1996*). PBA is founded on the use of probability boxes (p-boxes) combining probability and interval arithmetic. In PBA, variability may continue to be characterised by probability distributions, whereas knowledge uncertainty (bias) is described by intervals. P-boxes are used to generalise both these characteristics by placing interval bounds on cumulative probability distributions. A p-box is the class of distribution functions $F(x)$ bounded by two cumulative distribution functions $F_1(x)$ and $F_2(x)$ such that $F_1(x) \leq F(x) \leq F_2(x)$ for all x.

QSAR models can be viewed as an example. The prediction uncertainty for a typical regression model with point estimates can be estimated by internal or external (preferably) validation and reported as a probability distribution (*Sahlin et al., 2011*). While this expression of uncertainty will not completely cover the model uncertainty, i.e. how confident we are in the predictions, an external validation may give a partial estimate of bias. The calibration data for a model will also define its applicability domain, and models outside of this domain are not considered reliable (unknown bias). However, the specification of this domain is an uncertain issue in itself and other means to improve the reliability is to combine several models ('consensus modelling') or to combine with other sources of evidence (WoE). This illustrates the fact that knowledge uncertainty (bias) can be reduced, but also the fact that it is difficult to describe by precise probability distributions.

So far we have discussed uncertainty arising from variability and inherent randomness, that can be estimated and described by probability distributions, and uncertainty due to lack of knowledge (bias), that can be estimated and described by intervals. For both kinds of uncertainty it thus requires some information about the uncertainty to expect. Totally unexpected uncertainty (surprises) cannot be assessed with scientific methods, although sensitivity analysis applying different extreme scenarios can give information about the critical

parameters having the largest effect on a given safety assessment. It should also be noted that there is no difference in principal between animal models and non-standard methods when it comes to surprises.

Generally a quantitative assessment of an endpoint also requires a quantitative assessment of the uncertainty; as an interval, a probability distribution or a combination of the two. Often verbal statements such as 'high', 'low', 'medium', etc. are used, but such statements mean different things to different recipients. Similarly, an expert elicitation to estimate uncertainty will require a calibration among participants.

2.4.4 Uncertainty and Incomplete Evidence in Combined-Approach Predictions

In general, incomplete knowledge leading to scientific uncertainty can be addressed by (i) assessing and describing the uncertainty (including the determination of assessment factors); (ii) examining and describing the impact and implications of the uncertainty on the final conclusions of the work; and (iii) communicating both these elements in suitable terms understandable to the target audience. It is important to assess the uncertainty related to the methods and incomplete knowledge and describe and communicate it in a transparent way.

Uncertainties related to hazard identification, characterisation and extrapolation are generally taken into account during the derivation step in the determination of the no effect level and are covered by using default assessment (uncertainty) factors which may be modified case by case, based on the information available. The current approaches and assessment factors in DNEL derivation described in ECHA guidance documents provide a basis of estimating uncertainty. However, regulatory science would need to be developed to deal with uncertainty from WoE and non-animal approaches. The use of alternative methods including high throughput assays can introduce additional components of uncertainty regarding dose-response extrapolations and for use in hazard assessment. It should be noted that there is an opening for using such non-standard approaches to address the REACH standard information requirements by means of a WoE approach.

Uncertainty analysis and relevant methodologies have been developed or are currently being developed for risk assessment. For example, WHO/IPCS has developed approaches for exposure and hazard assessment (*WHO, 2008; WHO; 2014*). The principles outlined in WHO guidance documents would also apply in the use of non-test methods in general, although this has not been applied only qualitatively. In addition EFSA have identified the interlinked 'triad' of issues priority areas for updating guidance: (i) assessing and dealing with uncertainty; (ii) combining different lines of evidence; and (iii) biological relevance (i.e. adaptive verses adverse effects). Hence regulatory science developments are anticipated both from WHO and EFSA.

2.4.5 Final Comments

Regulatory science is applied science using evolving methodologies/approaches. Science may be incomplete yet regulatory decisions still must be made in spite of incomplete knowledge. Assessments of particular cases using the current state of the art approaches, including the associated uncertainty, inform decision-making but the final outcome may include other factors and policy considerations depending on the particular regulatory scheme.

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Disclaimer

The views expressed in this paper are solely those of the authors and the content of the paper does not represent an official position of the European Chemicals Agency.

2.5 Toward Mechanistic Validation

Thomas Hartung

2.5.1 Abstract

Today's mechanistic toxicology is effectively relying to large extent on methodologies which substitute or complement traditional animal tests. The biotechnology and informatics revolution of the last decades has made such technologies broadly available and useful. Regulatory toxicology has started to embrace these new approaches. However, for broader regulatory acceptance these methods need to be validated. Validation of new approaches in regulatory toxicology is commonly defined as the independent assessment of the reproducibility and relevance (the scientific basis and predictive capacity) of a test for a particular purpose. In large ring trials, the emphasis to date has been mainly on reproducibility and predictive capacity (comparison to the traditional test) with less attention given to scientific or mechanistic basis. Assessing predictive capacity is difficult for novel approaches, (which are based on mechanism) such as pathways of toxicity or the complex networks within the organism (systems toxicology). This is highly relevant for implementing *Toxicology for the 21st Century,* either by high-throughput testing in ToxCast/Tox21 project or '-omics'-based testing in the Human Toxome project (http://humantoxome.com). It is suggested that the mostly neglected assessment of a test's scientific basis, which moves mechanism and causality

to the foreground when validating/qualifying tests, can play a major role in the validation of novel mechanism-based tools. Such mechanistic validation faces the problem of establishing causality in complex systems. However, pragmatic adaptations of the Bradford Hill criteria, as well as bioinformatic tools, are emerging.

2.5.2 Introduction

Major validation efforts have delivered the evidence that new approaches in toxicology do not lower safety standards and can be integrated into regulatory safety assessments. In the US, especially the NAS vision report for a toxicology in the 21st century (Tox-21c; *NRC, 2007*) and its most recent adaptation by EPA for their toxicity testing strategy have initiated a debate how to create a novel approach based on human cell cultures, lower species, high-throughput testing and modelling. A systematic mapping of the entirety of pathways of toxicity, the Human Toxome, has been started (http://humantoxome.com; *Hartung & McBride, 2011; Bouhifd et al., 2014*). Emerging technologies and numerous initiatives are being created worldwide to promote their use to assess toxicity. This should lead to pathway based tests and ultimately to the integration of their results in a Systems Toxicology approach (*Hartung et al., 2012*). We have learned from the development, validation and acceptance of alternative methods for the creation of a new approach for regulatory toxicology. A multi-stakeholder process to develop a roadmap for replacing animal-based systemic toxicity testing has been started (*Basketter et al., 2012*). It identified Integrated Testing Strategies (ITS; *Hartung et al., 2013a*) and pathways of toxicity (PoT) based approaches (*Kleensang et al., 2014*) as most promising in accordance with Tox-21c.

To assist in the culture change and paradigm shift, it is important to establish a mutually beneficial dialogue among stakeholders in the current system. This dialogue will focus on quality assurance of the novel tools. Traditionally, this was attempted by formal validation. However, traditional validation has two principal problems:

- It is costly, takes a long time and is not amenable to change based on new developments in technology, as any change invalidates the validation;
- Validation is done using current, imperfect, traditional animal-based methods as the point of reference and thus cannot lead to a paradigm shift.

Therefore, a concept that assures quality without these limitations is necessary. The 'Center for Alternatives to Animal Testing' (CAAT) toxicity testing symposia touched on this issue, which was taken up in detail at a CAAT organised conference '*21st Century Validation for 21st Century Tools'* in July 2010. From that conference, a steering group was formed which includes representation from CAAT, EPA, FDA, the National Toxicology Program, the American Chemistry Council, CropLife America, the pharmaceutical industry, the Humane Society of the US, the Institute for *In-Vitro* Sciences and ILSI/HESI and others. The group has embraced the

concept of Evidence-based Toxicology (EBT) as a substitute for traditional validation (*Hartung, 2010*) and views the development of this concept as a prime opportunity to collaborate toward change in regulatory toxicology. This group promotes a private-public-partnership (the Evidence-based Toxicology Collaboration, EBTC, http://www.ebtox.com) between agencies and industry to promote quality assurance and implementation of new approaches (*Hoffman et al., 2014*). The EBTC was inaugurated on 10 March 2011 as a satellite activity to the 50th Society of Toxicology conference in Washington (*Zurlo, 2011*). A European branch followed one year later, started as a satellite to EuroTox in Stockholm, Sweden, 2012. CAAT entertains the secretariat for EBTC. While the costs for individual evaluations of new methods must be borne by their developers and promoters, a central steering and publically available repository for guidance and reference documents is necessary (similar to the Cochrane library for Evidence-based Medicine).

The secretariat assumes the following responsibilities:

- Central coordination of the steering group, the organisation of EBTC and the appointment of evaluation committees;
- A standing committee for horizontal EBT method development (meta-analysis, quality scoring tools, probabilistic risk assessment etc.);
- An Internet portal for guidance and reference materials;
- Public relations.

The opportunities and needs for quality assurance (*Hartung, 2007; 2009a; 2010; Leist et al., 2012*) have been a core interest of the EBTC from the beginning. The steering group of EBTC formed for example at the 2011 CAAT conference *21st century validation for 21st century methods* and the first EBTC conference hosted by EPA in 2012 (*Stephens et al., 2013*) resulted in a consensus paper on validation of high-thoughput methods (*Judson et al., 2013*) as used in ToxCast. Very often these discussions touched on the need for a mechanistic approach to testing that generates relevant evidence, which can then be compiled to inform decision-making. In a recent paper (*Hartung et al., 2013b*), we addressed this mechanistic thinking with respect to the problem of confirming a biological mechanism and using established mechanisms as the basis for validating our test systems. Thus, it is a discussion of biological causality in a field that is increasingly becoming aware of the complexity of the organism and embracing a systems toxicology approach. Here, several aspects, which are essential when embarking on mechanistic validation, shall be discussed.

2.5.3 Historical Background and Future Perspectives for Mechanistic Validation

The classical definition of validation was coined in 1990 at an ECVAM/ERGATT workshop:

> *"Validation is the process by which the reliability and relevance of a new method is established for a specific purpose"* (*Balls et al., 1990*).

The importance of the scientific basis was proposed by (*Worth & Balls, 2001*). The modular approach (*Hartung et al., 2004*), a consensus between ECVAM and ICCVAM, introduced this aspect of scientific validity and referred also to the prediction model:

> *"Validation is a process in which the scientific basis and reproducibility of a test system, and the predictive capacity of an associated prediction model, undergo independent assessment" (Hartung et al., 2004).*

The modular approach was embraced in the OECD guidance document on validation (*OECD, 2005*). The challenges to the current validation paradigm, such as the imperfections of the reference test, the inability to demonstrate that a new test is better than the reference test, the costs and duration of the current process, and its failure – to date – to be adopted to testing strategies, have been discussed elsewhere (*Hartung, 2007; Leist et al., 2012*). In addition, we have earlier stressed the opportunity that lies in this aspect of scientific basis (*Hartung, 2010; Hartung & Zurlo, 2012*).

Biomedical science addresses how living organisms work and how proper functioning can be disturbed or restored. When moving to a systems approach, this is all about mechanism, i.e., a level of resolution lower than the macroscopic and phenomenological view. It is about the 'how?' Toxicology has embraced a focus on mechanism for a couple of decades and we have termed it 'mechanistic', 'predictive', 'translational', etc. Some, when fearing that the promise to identify mechanism might be difficult to realise in practice, introduced 'mode-of-action' to allow for uncertainty in characterising mechanism. As defined in the US EPA draft, *Mechanisms and Mode of Dioxin Action*, mechanism of action is '*the detailed molecular description of key events in the induction of cancer or other health endpoints',* whereas mode-of-action refers to *'the description of key events and processes, starting with interaction of an agent with the cell through functional and anatomical changes, resulting in cancer or other health endpoints'.*[1]

The classical frameworks of Koch-Dale and Bradford-Hill serve to establish causality (*Hartung et al., 2013b*). Koch's postulates were aimed at giving unambiguous proof of causality for a pathogen causing a disease. When translated to physiology by Dale, the idea remained to request similar evidence as for pathogenesis of an infectious disease, which together makes the case of a linear causality of mediation of an effect. The problem is that few things in biology are linear and networked systems are too complex to provide certainty when interrogated, given that most experiments only remain valid if some variables are kept constant. Sir Bradford Hill (*Hill, 1965*), in contrast, gave a number of types and pieces of evidence that support causality without the assumption of a simple linear relationship. It is undoubtedly the more adequate framework for complex systems, in his case epidemiology, and, thus, for a systems toxicology approach.

1 - Available at http://www.epa.gov/ncea/pdfs/dioxin/nas-review/pdfs/part3/dioxin_pt3_ch03_oct2004.pdf

The beauty of the Koch-Dale approach lies in its straightforward guidance on which experiments to carry out to determine support causality. It asks, for a mediator (originally a disease agent; in Koch's case a microbial pathogen): Show that the mediator is present when the disease state forms and show that you can protect the organism by blocking its formation or action and that you can induce (or aggravate) the disease state by its (co-)application. Translated to the paradigm of *Toxicity Testing in the 21st Century: A Vision and a Strategy* (*NRC, 2007*) or Tox-21c, for a pathway of toxicity (PoT), this means: *show it, block it* and *induce it.* If these experiments agree, we are on a good track to confirming the PoT.

Validating a mechanism in toxicology means establishing the causality between toxicant and hazard manifestation and identification of how it happens. Together the two approaches (Koch/Dale and Bradford Hill) help to support (not prove) causality, but only by establishing causality between toxicant and hazard. They can be used for confirming a mechanism when applied to the mediating events. This means that, in principle, for each and every event of a PoT we need to establish causality. Neither framework was developed for causality in toxicology and Bradford Hill was very careful to offer his criteria as a comparative standard, i.e., it is only valid if there is no better plausible alternative explanation of the effect. In our case, the comparative standard would be the scientific evidence supporting a specific mechanism. In order to maximise existing knowledge and minimise subjectivity in establishing standards, a central, frequently updated repository of accumulated mechanistic knowledge is required.

Notably, there is no institution for collecting the evidence for a certain mechanism to be responsible for causing an effect, nor is there a repository for retrieving the information once accumulated. This is exactly what the Human Toxome project (*Hartung & McBride, 2011; Baker, 2013; Bouhifd et al., 2014*) attempts for toxicology, which admittedly is only a small part of the life sciences. It is based on the notion that groups of toxicants leading to similar hazard manifestations are likely employing the same or similar mechanisms (pathways of toxicity), resulting in the same disturbed physiology. An alternative view might be that there are only a certain number of meta-stable physiological states a disturbed biology can assume before collapsing, and they are linked with some probability to particular hazard manifestations. In conclusion, validating the mechanism of a (group of) toxicant(s) is the basis for mechanistic validation of tests that identify those toxicants.

Mechanistic validation (*Figure 2.5*) might serve the process of moving away from correlation of phenomena toward the molecular description of pathways (*Hartung & McBride, 2011*). Put simply, the steps that should be part of mechanistic validation are:

- Condense the knowledge of biological/mechanistic circuitry (in the absence of xenobiotic challenge) underlying the hazard in question;
- Compile evidence that reference chemicals leading to the hazard in question perturb the biology in question, i.e. mainly pathway identification by using reference substances in valid(ated) models and experimental proof of their role;

- Develop a test that purports to reflect this biology;
- Verify that toxicants shown to employ this mechanism also do so in the model;
- Verify that interference with this mechanism hinders positive test results.

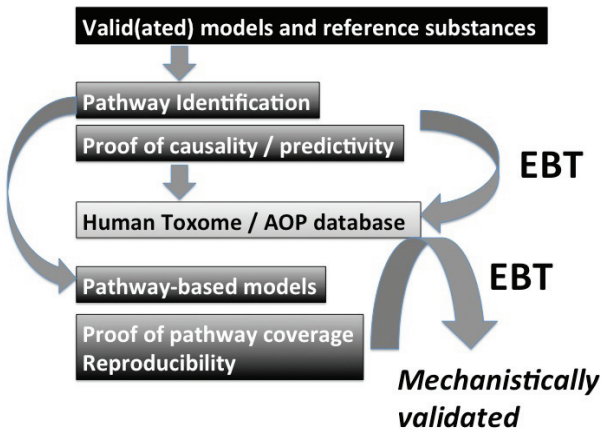

Figure 2.5 *The Mechanistic Validation Scheme for test systems with a possible role for Evidence-based Toxicology (EBT) type of assessments (from Hartung et al., 2013b, reproduced with permission).*

This still proves mediation at every step, but with plausibility and the respective experimental underpinning. First, we would show that a certain mechanism is involved and whether it is necessary and/or sufficient or aggravating. Then we can ask whether a given test reflects this mechanism. In contrast to traditional validation, this will not require testing of large numbers of new substances. Rather, it entails identifying toxicants that result in the same hazard in question and showing that they employ the mechanism in the pathway-based test as the chemical used to deduce the PoT. We should keep in mind that, unlike epidemiology, where the conceptual framework by Bradford Hill originates, toxicology can typically use experimental interventions, though with all the limitations of these models.

A key question is: how should we assess a chemical lacking hazard information in the absence of mechanistic information? Can we use the following information to test a chemical whose mechanism of action is unknown? We will need (i) knowledge of biological/mechanistic circuitry relevant to xenobiotic challenge; (ii) tests that purport to reflect key mechanisms in biology; and (iii), verification that toxicants that have been shown to employ one or more of these mechanisms also do so in the test system. This might be done even in a relatively small part of the chemical universe; we have termed this approach "*test-across*" (similar to *read-across*; *Hartung, 2007*), i.e., creating local applicability domains by showing that (structurally) related substances are correctly identified.

We have stressed (*Hartung, 2009a*) that the call for evidence-based toxicology (EBT) has two roots – Philip Guzelian's group's proposal for a more rigorous approach to causation of chemical effects (*Guzelian et al., 2005*), and ours (*Hoffmann & Hartung, 2006*) seeking new approaches to method evaluation. The proposal for a mechanistic validation fuses the two concepts and uses causation to evaluate methods. By ascertaining mechanistic validity (*Hartung, 2010; Hartung & Zurlo, 2012*) we can qualify/assess (avoiding the term 'validate', which is typically used for the correlative traditional validation approaches) both the components of ITS (*Hartung et al., 2013a*) and high-throughput tests (*Judson et al., 2013*).

We earlier stressed that the main similarity of Evidence-based Medicine and EBT is actually clearer when viewing a toxicological method as a diagnostic test (*Hoffmann & Hartung, 2005; Hartung, 2010*). It is interesting that this discussion has been largely driven by test accuracy and very little by mechanism, which is quite different to biomarker qualification.

By suggesting EBT as a starting point for method validation for Tox-21c and thus for mechanistic methods, we are facilitating convergence on the basis of causation. The next logical step is establishing the mechanistic basis of assays used in the HTS. This is a tremendous opportunity for the EBT Collaboration. EBT incorporates, from its role model Evidence-based Medicine, the overarching evidence-based principles of transparency, objectivity, and consistency. These defining characteristics assist any process, whether based on mechanism or correlation, in surviving peer scrutiny. EBT offers more than the actual result of a systematic review and creates the possibility of continuous improvement in the light of additional evidence. A high-quality assessment of the state of the evidence will always also be an assessment of the uncertainty and the limitations of the data. This is, by itself, as valuable as the actual condensation of the available evidence.

Validations of new methods have traditionally been carried out by comparing them to the tests they aim to replace, with the problematic assumption that pre-existing tests represent a gold standard. As the results of the reference test are classifications, the classified toxicants are the point of reference. An important ECVAM workshop discussing points of reference for validation (*Hoffmann et al., 2008*) suggested a move to a composite point of reference, where all knowledge of toxicants is used to create the correct classification. This allows, for example, sorting false-positive and –negative results. The goal is no longer to reproduce the traditional test with all its shortcomings but to define what an ideal test would identify. If we now move in this direction (*Hartung, 2007*), we will have to build a consensus on a relevant mechanism and its contribution to hazard manifestation. The Human Toxome project aims to develop the process for doing exactly this. To put it simply: no agreed mechanism, no mechanistic validation. The Human Toxome project does not aim to confirm known/presumed pathways, but to be open to new causal links. We would quickly run out of pathways if we focused only on those already known. We also would only reinforce our biases, overstressing what we *believe* to know compared to what we *want* to know. For this reason, the project begins with

untargeted analyses of chemically induced metabolites and transcripts. By associating the patterns of change (i.e., the signatures of toxicity (SoT) to the pathways of toxicity (PoT)), the noise common to all of systems is eliminated. The two orthogonal technologies, as well as replicates and concentration/response relationships around the thresholds of adversity, further focus PoT identification.

It is important to keep in mind that such a mechanistic validation does not necessarily need reference chemicals, nor does it rely on animal data as gold standards. In principle, it can facilitate the shift to human biology under Tox-21c – for this purpose, the validation can rely, for example, on the use of a cell or tissue's own biochemistry (agonists, antagonists, enzymes, hormones, etc.) and to show biological relevance of the pathway in the test system, besides the use of known xenobiotic disrupters (toxicants, pharmacological as well as scientific inhibitors) of a mechanism, to show merit of the assay.

2.5.4 Conclusions

Mechanistic thinking opens new avenues for assessing the performance of test methods. Such thinking bases our confidence not on correlation but on the accumulated knowledge of how a particular exposure leads to particular effects. This approach requires certainty in our deduction of mechanism and becomes more difficult as we acknowledge the complexity of systems and our lack of understanding thereof. If we assume that causation is linear, we have a simple approach to prove it (Koch-Dale). If we take complexity into account we are left with ascertaining a relationship (Bradford-Hill). As we increase our understanding of the system we are studying we can begin to model and carry out virtual experiments to understand causality and verify these predictions by experiments.

This opens up the possibility of a mechanistic validation, especially where the type of information generated does not directly correspond to a high-quality point of reference. This approach entails the danger that it is based on our current level of understanding. When scientific paradigms change, we have to review what we concluded from the old concepts, but it might still be better to base our regulatory science on the current understanding of pathophysiology and not on pure correlations.

What does this mean for the validation process? The key change will be the introduction of a module for scientific relevance into the 7-step modular approach (*Hartung et al., 2004*). We do not suggest making this a new module 8 (scientific relevance) but rather to add it as a new option to existing module 5 (predictive relevance). The latter would become module 5a with scientific relevance becoming module 5b. As stressed earlier (*Judson et al., 2013*), for high-throughput methods it will be necessary to compensate for often lacking information on inter-laboratory reproducibility (module 3), as often no adequate facilities for ring trials are available,

but within-laboratory variability is low anyway. Again we might consider that strengthening our assessment with mechanistic relevance might help here, though it provides a different type of confirmation. It might be promising to start formally validating the mechanistic basis of assays in the current large scale high-throughput testing programs in toxicology (ToxCast and Tox-21 project, see section 5.2.2). Similarly, mechanistic validation might help to quality-assure the Adverse Outcome Pathways (AOP) currently developed by OECD.

The obvious practical problem with Mechanistic Validation is that it depends on our current understanding of the system and the identified mechanisms. Some might argue that we need full understanding of the system, which we can never attain. However, being aware that we can only approximate (model) the system, we can test the predictivity for some, but not all areas, where we *do* have a point of reference. Deduction and annotation of mechanisms are key prerequisites for a Mechanistic Validation. Creating such a repository, or knowledge base, of pathways of toxicity is the goal of the Human Toxome project. Although its governance has not been established, consensus on the process and types of information to be compiled is emerging. A t[4] (Transatlantic Think Tank for Toxicology) workshop on this topic was held in Baltimore in October, 2012 (*Kleensang et al., 2014*).

The EBT toolbox lends itself to a Mechanistic Validation as it offers processes to compile and evaluate evidence objectively and transparently (*Hartung, 2010*). It might become the sparring partner for new method development and quality assurance. However, it might as well be conceived that the traditional validation process embraces the same approaches to tackle the challenge of validation of 21st century technologies. Beside the technical development of new approaches, we need both conceptual steering and an objective assessment of current practices by evidence-based toxicology (Hartung, 2009a; b).

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2.6 Read-Across as a Basis for One of the SEURAT-1 Proof-of-Concepts and an Overview of the Outcome of the SEURAT-1 Read-Across Workshop

Terry W. Schultz

2.6.1 Background

Read-across is the process of assessing a toxic endpoint of an untested substance based on the results for the same endpoint for one or a few tested substances considered to be similar (*Dimitrov & Mekenyan, 2010*). As such, it is considered a non-testing method for filling data gaps based on an analogue or chemical category (*Van Leeuwen et al., 2009*). Read-across is a predictive technique for chemical limited used at the turn of the century. Within the EU, it was primarily applied for hazard assessment of chemicals in the context of harmonised classification under Regulation 1272/2008 (CLP) and its predecessor Directive 67/548/EEC. There was a lack of clear guidance on how to apply read-across and it was made on a case by case basis using expert judgment. However, since 2010, much has been written about the use of read-across as a predictive tool for REACH and CLP. This recent interest in the process of read-across comes largely from the fact that it is the primary non-test method for completing submissions for REACH (*Patlewicz et al., 2013*).

Read-across, under REACH, is addressed in Annex XI, 1.5 '*Grouping of substances and read-across approach*' (*European Commission, 2007*). In Annex XI, 1.5 it states substances who are likely to be similar or follow a regular pattern as a result of structural similarity may be considered as a group or 'category' of substances and, within the category, the read-across approach can be applied. Key to the REACH application of read-across is the statement, '*If the group concept is applied, ... adequate and reliable documentation of the applied method shall be provided*' (*European Commission, 2007, p. 121*). This means that after the read-across is carried out, it has to be decided during the assessment whether the case is convincing enough to accept the prediction. While the acceptance of read-across cases is made according to a standard procedure, in the end, the evaluator must be convinced in a scientifically credible manner based on the theory and supporting data provided. Key to acceptance is addressing the uncertainties inherent to the read-across. These issues have recently been discussed (*Cronin, 2013*).

Read-across reduces testing, especially animal testing, by using test-based endpoint information for one or several substances (i.e., the source chemicals) to predict the same endpoint for a 'similar' untested substance (i.e., the target chemical). In an effort to address

uncertainty factors, it is often applied in a weight-of-evidence approach (*Cronin, 2013*).

The most critical issue in any read-across exercise is the justification of analogue(s) selection for the read-across (i.e., explaining the criteria of chemical similarity on which the selection is based). This is a recurring issue because similarity is sometimes assessed in a subjective manner. Therefore, in order to gain acceptance of any read-across prediction, it is essential to explain the basis for similarity between the target chemical and potential source chemicals in a robust and reliable manner (*Enoch & Roberts, 2013*). Common chemical features on which toxicological read-across is based include:

- Physico-chemical and molecular properties;
- Substituents, functional groups and extended structural fragments;
- Two-dimensional (2D) molecular similarity (i.e., statistical similarity based on graph theory).

Common biological features on which toxicological read-across is based include:

- Bio-modification (e.g., metabolism, activation or degradation);
- Structural alert for chemical-biological interaction;
- *In vitro* data relevant to the read-across endpoint(s);
- Adverse Outcome Pathways (AOPs).

A second critical issue in any read-across exercise is assessing the uncertainty associated with the outcome of the exercise (*Blackburn & Stuard, 2014*). Uncertainty in a read-across prediction can be characterised by a number of criteria including; (i) the number of analogues in the source set; (ii) the concordance with regard to the data; and (iii) the severity of the hazard (*Blackburn & Stuard, 2014*). The goal here is to explain the type and degree of uncertainty for each endpoint-specific read-across. In order to be consistent in the manner in which uncertainty is assessed in read-across, an assessment framework is often used. Generally speaking, the aim of these frameworks is to:

- Describe the rationale of the read-across in a transparent manner;
- Document the logic leading to the prediction so it can be recreated by the reader;
- Separate data uncertainty from read-across uncertainty;
- Clarify the roles of endpoint specific and endpoint non-specific factors in the assessment.

While read-across is still a young concept, there is emerging an agreement on what entails minimal documentation of a read-across exercise. There is growing agreement that documentation of a read-across should include a:

- Statement of the read-across hypothesis;
- Statement of the justification for the read-across hypothesis (i.e., basis of the similarity);
- List of all the substances included in exercise (i.e., the target chemical and the source chemicals);
- List of identity information (e.g., name, CAS number and structure) of all substances included in the exercise;
- List of the endpoints that are to be read-across;
- Data matrix;
- Statement of uncertainty associated with the read-across;
- Statement of the conclusions of the read-across exercise.

2.6.2 The SEURAT-1 Read-Across Workshop

Twenty-three invited experts from Europe and North America, including representatives from academic, governmental and industrial institutions, attended the **SEURAT-1** workshop entitled: *'The read-across case study for safety assessment contributing to the SEURAT-1 Proof of Concept'* that took place at the Joint Research Centre (JRC) in Ispra, Italy on 29–30 April 2014. The workshop was an initiative of the **SEURAT-1** Safety Assessment Working Group (see section 4.11.8), co-led by Derek Knight (ECHA) and Andrew White (Unilever). The workshop was coordinated by Elisabet Berggren (JRC) and chaired by Karen Blackburn (Proctor & Gamble). The read-across exercises discussed at the workshop and the recommendations and conclusions of the workshop focused on the repeated dose target organ systemic toxicity, as the **SEURAT-1** Research Initiative is aiming toward replacement of repeated dose animal testing used for human safety assessment. As Cosmetics Europe is co-funding **SEURAT-1** together with the European Commission (FP7), it was considered an advantage if the chemical categories proposed at the workshop could be related to cosmetic or cosmetic-like ingredients.

The purpose of the two-day event included establishing some guiding principles to be used for the **SEURAT-1** Proof-of-Concept to apply a mode-of-action based hypothesis supported by data from *in silico* and *in vitro* methods for chemical safety assessment, which included:

- Agree on the most likely read-across scenarios;
- Select chemical categories suitable for use in proofs-of-concept for the different scenarios identified;
- Identify likely lead chemicals and potential category members to be targeted for data acquisition with the express purpose of supporting the read-across exercises identified in aim 2.

These aims were largely met and a series of recommendations and conclusions were reached. The key outputs of the workshop were a framework for addressing the main read-across scenarios, and a strategy for putting data into the framework.

Four of the most likely scenarios refined and examined by the workshop participants were:

1) Chemical similarity of direct-acting toxicants with a similar mechanism of action (no metabolism or metabolism not a driver of toxicity);

2) Chemical similarity involving metabolism-driven toxicity (resulting from exposure to parent toxicants with similar metabolites);

3) Chemical similarity of toxicants with no obvious reactive or specific mode-of-action (generic effects of low potency);

4) Chemical similarity of toxicants with overt toxicity and a presumed mode-of-action.

Each of the four scenarios was discussed by the participants in a round-table format. Particular attention was given to identifying chemical pairs or chemical categories that may be likely candidates for the four read-across proof-of-concept exercises.

It was agreed that a chemical category rather than a single pair of analogues was the better exercise. There was agreement that at least one of the read-across analogues or source chemicals must be well-studied and have high quality *in vivo* data (i.e., an effort to reduce uncertainty associated with the data). The latter point would be met by using **SEURAT-1** standard reference compounds (gold compounds, see section 4.11.3). The participants further agreed that there is, at minimum, a need for clear and endpoint-related definition of the basis for the chemical similarity of the category; some indication of biological similarity and, if possible, dissimilarity is also preferred.

The experts recommended at least two chemical categories for each read-across scenario. The tentative outcome of their recommendations is presented in *Table 2.2*.

Table 2.2 *Recommendations for chemical categories covering the four scenarios of the* ***SEURAT-1*** *read-across case study.*

Read-Across Scenario	First Chemical Category	Second Chemical Category
Direct-acting toxicants with a similar mechanism of action	Perfluorinated acids	Phthalates or pesticides
Metabolism-driven toxicity	α,β-Unsaturated alcohols	Halogenated solvents (ie.g., trichloroethane tertrachloroethane etc.)
Toxicants with no obvious reactive or specific mode-of-action	Saturated aliphatic alcohols	Propylene glycol ethers
Toxicants with structure similarity but markedly different potency	Short-chain carboxylic acids	Alkyl-substituted phenols

It was further agreed that each category should:

- Be based on a hypothesis;
- Include at least one 'ringer member' (i.e., one analogue hypothesised to be an outlier to the category);
- Include a limited number of chemicals (i.e., five or more substances but less than twenty).

Categories where specific analogues were proposed and discussed included β-unsaturated alcohols, saturated aliphatic alcohols and short-chain carboxylic acids.

In the case of β-unsaturated alcohols, it is hypothesised that the alcohol will undergo metabolic transformation to the corresponded α,β-unsaturated aldehyde via cytosolic alcohol dehydrogenase (*Bradbury & Christensen, 1991*). This transformation would subsequently lead to liver fibrosis in repeated dose testing (*Landesmann et al., 2012*). The positive *in vivo* tested read-across source chemical allyl alcohol (1-Propen-3-ol) [107-18-6] is a secondary allylic alcohol of the generic structure 1-alken-3-ol. Other analogues recommended for this category are listed in *Table 2.3*. The structural relationship (as an indicator for chemical similarity) between allylic alcohol, the source compound, and the potential target substances, as well as among the target substance are demonstrated in *Figure 2.6*.

Table 2.3 *Recommended compounds to be used as analogues (target compounds) to the source compound allyl alcohol in a read-across case study covering the scenario 'chemical similarity involving metabolism-driven toxicity'.*

No.	Name	CAS No.	Type of Alcohol	General Structure
1	1-Buten-3-ol	598-32-3	sec. allylic	1-alken-3-ol
2	1-Penten-3-ol	616-25-1	sec. allylic	1-alken-3-ol
3	1-Pentyn-3-ol	4187-86-4	sec. propargylics	1-alkyn-3-ol
4	1-Propanol	71-23-8	prim. saturated	alkan-1-ol
5	trans-2-Penten-1-ol	1576-96-1	prim. allylic	2-alken-1-ol
6	2-Buten-4-ol	6117-91-5	sec. allylic	2-alken-4-ol
7	3-Methyl-1-buten-3-ol	115-18-4	tert. allylic	1-alken-3-alkan-3-ol

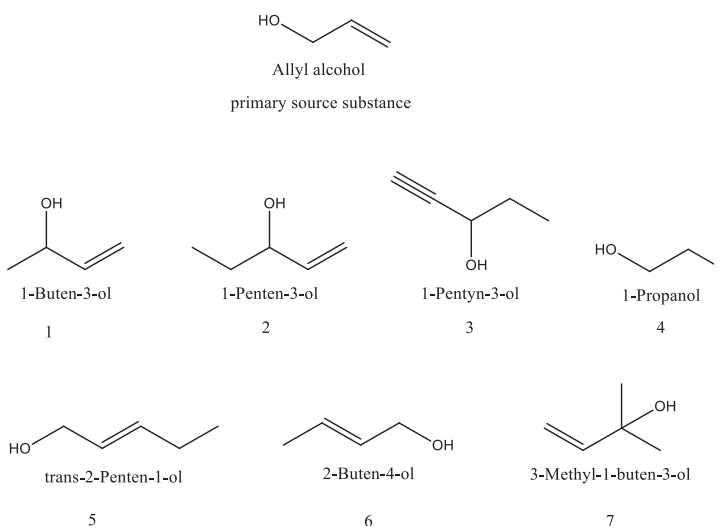

Figure 2.6 *Source compound and potential target analogues for pro-electrophilic alcohols and liver fibrosis.*

Compounds 1 – 3 are *a priori* predicted with high confidence to be members of the chemical category experimentally defined by the source compound, allylic alcohol, to elicit liver fibrosis in repeated dose testing. Chemical 4, 1-propanol, is the ringer chemical (a non- β-unsaturated

alcohol) and is predicted *a priori* with high confidence to be an outlier to the category. Chemicals 5 and 6 are *a priori* predicted with low confidence to be members of the chemical category defined by the source compound allylic alcohol. Chemical 7, 3-methyl-1-buten-3-ol, because it lacks the ability to be metabolised to its corresponded α,β-unsaturated aldehyde via alcohol dehydrogenase, is *a priori* predicted with low confidence to be an outlier to the category.

It is proposed that *in vitro* molecular screening assays with heptocytes or hepatocyte-like cells, as well as *in chemico* reactive screening and *in silico* models, can provide 'new approach' data. These data may be used to improve the robustness of the read-across case and reduce the uncertainty of the prediction by refining the chemical similarity and providing endpoint-directed evaluation of biological similarity.

Saturated aliphatic alcohols provide an excellent example where read-across may clear a number of derivatives for the repeated dose endpoint. In this case, the category has no obvious chemical reactive, no obvious bioactive and a high no observable effect concentration (e.g., ≥ 500 mg/kg/day) for repeated dose tested analogues. ToxCast has data on more than a dozen longer chained (≥ C6) saturated alcohols. Within ToxCast, these alcohols are among the 'least promiscuous chemical classes' with only 0.1-0.3% of the ToxCast assays showing any activity at the highest concentration tested and none of the active assay being associated with specific bioactivity. Since there is *in vivo* repeat dose data for at least one analogue, as well as ToxCast *in vitro* molecular screening data, *in chemico* reactive screening data and *in silico* model data for members of the category, this chemical category may be cleared without further testing in an *in silico* exercise.

The participants felt that different hypotheses can be formulated for the remaining six chemical categories. Based on these hypotheses, different source chemicals and target chemicals can be identified. Subsequently, intelligent testing schemes can be formulated, with the specific aim of gathering 'new approach' data, especially within the **SEURAT-1** Research Initiative. The idea is that the new approach data provided may be used to improve the robustness of the read-across cases and reduce the uncertainty of the predictions by refining the chemical similarity and providing endpoint-directed evaluation of biological similarity.

2.6.3 Conclusions

Read-across is the near-term solution to filling *in vivo* data gaps without additional *in vivo* testing. It is an effective bridging methodology which takes us from risk assessment based on *in vivo* animal testing to risk assessment based on alternative methods. Addressing seminal issues, such as 'similarity' and 'uncertainty', will aid in providing consistency, efficiency and transparency to read-across exercises. Integration of additional empirical 'new approach' data (e.g., *in vitro, in silico*, '-omics') should strengthen confidence in read-across assessments and ultimately allow us to expand actionable chemical domains. Designing and conducting

a series of proof-of-concept read-across exercises, such as those proposed here, will assist in providing knowledge and insight on how to provide evidence to substantiate read-across predictions, especially for regulatory acceptance. Additionally, integrating these read-across exercises with selected *in vitro* and *in silico* test systems being developed under the umbrella of the **SEURAT-1** Research Initiative will provide proof that the mechanistic understanding of toxicological being developed within the different aspects of the **SEURAT-1** work programme has regulatory applications.

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3 PROVING THE SEURAT-1 RESEARCH STRATEGY

Elisabet Berggren, and the principal investigators of the **SEURAT-1** proof-of-concept case studies.

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

Albert Einstein

3.1 Introduction

Elisabet Berggren

Chapter three of each **SEURAT-1** Annual Report has been dedicated to the development of the SEURAT strategy. In Volume 1, the **SEURAT-1** vision and strategy was outlined as a backbone to all **SEURAT-1** activities (*Whelan & Schwarz, 2011*). The strategy was then further developed into the **SEURAT-1** objectives, including the idea of proving the **SEURAT-1** concept in Volume 2 (*Whelan & Schwarz, 2012*). In Volume 3, the proof-of-concept was expanded to theoretical, systems and regulatory application levels (*Whelan & Schwarz, 2013*). During this last year, case studies have taken shape and today concrete work on how to prove **SEURAT-1** concepts is in progress in the laboratories and offices of **SEURAT-1** research partners. This activity was not originally foreseen when setting up the individual projects, but by developing trust and fruitful collaboration between **SEURAT-1** partners, the vision become a common basis for further commitment, and all projects are now contributing to proving **SEURAT-1** concepts.

3.2 SEURAT-1 Proof of Concepts

Elisabet Berggren

The four **SEURAT-1** objectives are:

- 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;
- Demonstration of proof-of-concept at multiple levels (theoretical, methodological, application);
- Provide the blueprint for expanding the applicability domains - chemical, toxicological and regulatory.

The multiple level proof-of-concept case studies were established based on the third objective. The understanding needed to apply a toxicological mode-of-action framework for chemicals safety assessment can be split into three steps. Firstly, it is necessary to make a theoretical hypothesis of what occurs when a chemical enters the body and starts a biological cascade

of events from molecular initiating events (MIE) to adverse outcomes (AO), that is, disease in a human. The hypothesis can be made based on existing knowledge, but once an adverse outcome pathway (AOP) construct that describes the sequence of events from MIE to AO is developed, it may need further elucidation and experimental proof. Such studies on the development, refinement or validation of AOP constructs within **SEURAT-1** are considered to be level 1 proof-of-concept case studies. Secondly, a toxicity prediction model based on the hypothesis should be developed. This model should integrate different experimental and computational methods and tools to predict an AO. These exercises are considered **SEURAT-1** level 2 proof-of-concept case studies. The AO prediction will then be a building block in safety assessments that include quantitative margins to protect human health and environment (*Figure 3.1).* These application scenarios in the context of safety assessment are considered as level 3 proof-of-concept case studies.

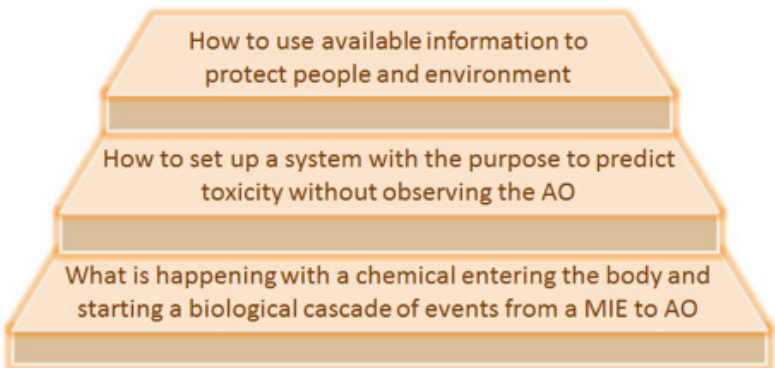

Figure 3.1 *Basic questions to be answered when linking interactions between chemicals and biological targets with the prediction of adverse outcomes in the context of safety assessment.*

AOP constructs are the starting point in this endeavour and they are discussed and developed in the Mode-of-Action Working Group, one of the **SEURAT-1** cross cluster activities (further details are give in section 4.11.5). Some AOPs are further refined and confirmed in project deliverables or in Level 1 case studies (see below). In principle, the first proof-of-concept level has already been achieved within **SEURAT-1** by the development of the three theoretical AOPs for the three major liver AOs, which are fibrosis, steatosis and cholestasis. The rationale behind AOP selection and development, as well as the practical approach of combining them, is described in detail in section 4.2. Activities to further elucidate or quantify the steps in these pathways are on-going and, as in any AOP development, continuous progress of refinement and correction must be considered. Drug-induced cholestasis, developed as a theoretical concept, is currently subject to a level 1 case study (see section 3.3 below).

AOP knowledge can be applied in different ways to predict toxicity. A complete AOP construct may be necessary to achieve biological understanding, but may not be necessary for setting up a prediction model. It could be sufficient to choose a key event common for many pathways, and then predict general toxicity affecting many organs simultaneously, for example mitochondrial toxicity. Alternatively, if an organ-specific key event can be identified as common for several major pathways, this could be further studied to predict target organ toxicity (*Figure 3.2*). The second case was illustrated for neurotoxicity in the report from the March 2013 **SEURAT-1** workshop in Ispra (*Landesmann & Vinken, 2013*; see Volume 3, section 4.10.5.6).

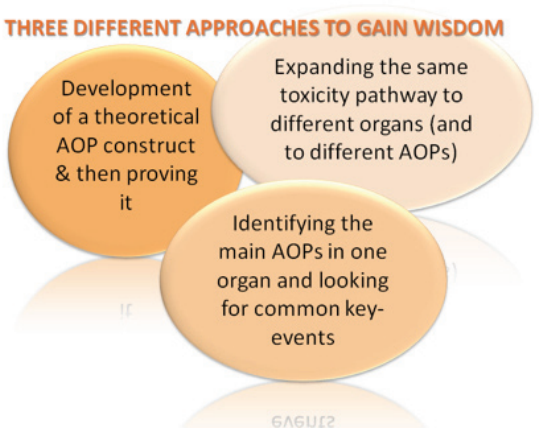

Figure 3.2 *Options for applying AOP knowledge to predict toxicity.*

When setting up a system for predicting toxicity, it is important to first identify the prediction goal (*Figure 3.3*). This sounds trivial but after going through the exercise, our experience shows that it is difficult to define a prediction goal related to AO, and then to maintain focus on the prediction goal. Sometimes exercises may be enlarged to include more basic mechanistic investigations, which might be relevant scientifically but do not directly assist in predicting toxicity. The next step in the **SEURAT-1** strategy is to describe the mechanistic basis, and then to select the chemicals anchoring the mechanistic basis, or a certain key event in the AOP to the AO. The last step is how to predict an *in vivo* quantitative point of departure that is necessary for the application to safety assessment – the third proof-of-concept level.

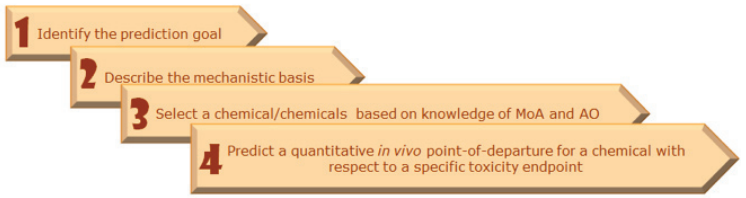

Figure 3.3 *The steps for designing* ***SEURAT-1*** *proof-of-concept case studies.*

The current six case studies at the second proof-of-concept level (dedicated to the development of integrated testing strategies) have the following prediction goals:

1. Liver fibrosis
2. Liver steatosis
3. Liver and kidney toxicity (organ specific AOPs)
4. Liver and heart toxicity (non-organ specific or 'general' AOPs)
5. Mitochondrial toxicity (non-organ specific or 'general' AOPs)
6. Liver/ non liver toxicity

The focus is concentrated on repeated dose liver toxicity, as *in vitro* methods using human hepatocytes or cell-lines were developed in many projects and because the theoretical AOP exercise (level 1) was focused on the major hepatotoxicity pathways, thus creating a basis for level 2. In addition, the level 1 case study on liver cholestasis could be developed in the future to include a prediction model for cholestasis. This would complete the set of the three major liver AOPs at the second proof-of-concept level. The different case studies can be interconnected: by selecting common chemicals certain logic patterns could be expected:

- A chemical shown to be positive in 1 or 2 should also be identified as hepatotoxic in 3;
- A chemical shown to be positive in 1, 2, 3 or 4 should also be identified as hepatotoxic in 6;
- A chemical shown to be positive in 1, 2, 3, 4 or 6 should (with high probability) be identified as mitochondrial toxic in 5, as mitochondrial damage is a key event in the major AOPs investigated, but can also be considered as leading to general toxicity;
- A known non-hepatotoxic chemical should be negative in all case studies looking at liver.

Additional questions to be considered when evaluating experimental outcomes from case studies, could include: is a non-hepatotoxic chemical also negative in the kidney (3) and heart (4), and if not, which AOP was triggered to cause toxicity? Is it negative in 5?

It is interesting to observe the range of methodologies used in the six case studies:

1. Liver fibrosis → complex hepatic microfluidic bioreactors composed of genetically modified and/or stem-cell-derived hepatocytes, hepatic stellate cells and sinusoidal endothelial cells;
2. Liver steatosis → 2D and 3D culture of HepaRG; mathematical modelling is used to predict effects on human liver by extrapolating the *in vitro* results;

3. Liver and kidney toxicity (organ specific AOPs) → Transcriptomics data is extracted from existing databases for substances known to cause liver and kidney toxicity by certain modes-of-action. Transcriptomics expressions for identified biomarkers are reproduced in *in vitro* systems, and then structurally related chemicals are tested to observe whether the same biomarkers are observed. The transcriptomics data is used to read across a known mode-of-action from data-rich source compounds to data-poor structurally related target compounds;

4. Liver and heart toxicity (non-organ specific or 'general' AOPs) → Transcriptomics expressions are used to predict general toxicity in compounds showing similar expression patterns without a prior knowledge of mode-of-action;

5. Mitochondrial toxicity (non-organ specific or 'general' AOPs) → development of an *in silico* profiler, composed of chemotypes, to identify compounds with the ability to induce mitochondrial toxicity;

6. Liver/ non-liver toxicity → A mode-of-action-based classification model aimed at distinguishing between hepatotoxicants and non-hepatotoxicants using *in vitro* HepaRG data achieved through high-throughput techniques.

The chemicals tested are selected to fit the prediction goal of each case study. The number of chemicals tested within a study varies due to the different systems applied. As a basis, the **SEURAT-1** Gold Compounds with known modes-of-action and target organs are used for the testing phase and to calibrate the predictive power of the method. In case studies 3, 5 and 6, a much larger set of chemicals was selected based on knowledge collected in the public domain. **SEURAT-1** Gold Compounds were still included as reference compounds and also to enable comparison of the results of the different case studies.

During level 2 case studies discussions at the fourth **SEURAT-1** Annual Meeting (Barcelona, 5-6 February 2014), it was agreed that it would not be useful to harmonise the endpoints between *in vitro* experiments performed in the different case studies; the systems vary too greatly. However, it was agreed that clear explanations of why particular read-outs were selected to meet the prediction goals were needed.

Another aspect discussed was the exposure protocols used in the experimental set-ups of the case studies. A relevant exposure scenario in the *in vitro* system must be chosen with the assumption that it is realistically mimicking what is happening to a cell in body tissue as an upstream event to *in vivo* repeated dose effects. An oft-used approximation has been that a one dose exposure (C_{max}) large enough to induce an observable effect in the *in vitro* system could be predictive to AO. It may be more realistic to continuously expose the biological system to a constant low concentration ($< C_{max}$). The integration of the low concentration over the time of exposure ('the area under the curve') will achieve the repeated dose effects. In

the latter case, assumptions about the clearance rate of the studied chemical must be made in order to select an appropriate dosing frequency that achieves a constant concentration in the cell. Additionally, read-out time points must be related to the timescale of the studied event (in relation to the chain of events in the AOP) and, if the effect is dependent not only on concentration, also to the time of chemical exposure. Another important experimental factor is the chemical concentrations chosen in the *in vitro* system and how they are related to *in vivo* exposure, i.e. an estimate of the concentration of the chemical reaching the target organ compared to the external dose. At the fourth annual meeting it was agreed that each case study description must include a rationale on the exposure treatment chosen. Case study researchers were strongly encouraged to use toxicokinetics reasoning when setting up experiments, and they were invited to request advice from the **SEURAT-1** Biokinetics Working Group. It was decided that the exposure protocols would not be harmonised between the case studies. This is due to the differences in experimental set-ups and also because there would be several relevant hypotheses for how to logically describe an *in vivo* repeated dose exposure in an *in vitro* study.

The results of the level 2 case studies (described in more detail in sections 3.4.1-3.4.6) will be presented at the next annual meeting, and the final reports will be summarised in the next Annual Report. The aim of each predictive toxicity case study is to fulfil its own prediction goal, and thus contribute to the **SEURAT-1** second proof-of-concept. An additional outcome of the predictive toxicity studies is to contribute results to the safety assessment of chemicals, which is the level 3 proof-of-concept. At the application level, all available information must be used to assist in assessing the safe use of a chemical. Varying weight can be given to this information, but with the ultimate aim of improving consumer/worker safety in a sustainable manner.

When making a chemical risk assessment for repeated dose target organ systemic toxicity, it is necessary to determine the lowest observed adverse effect level (LOAEL) *in vivo*. This could be done by looking at each target organ after excluding any general toxicity that would probably affect all organs, allowing enough exposure in dose and time. The idea could be to first exclude general toxicity pathways that might be represented by predicting certain typical key events, e.g. substantial mitochondrial toxicity. If the internal exposure causing general toxicity is considered low enough, it would be necessary to investigate whether more specific organ AOPs would be relevant for determining toxicity *in vitro*. The LOAEL *in vitro* must then be translated into a LOAEL *in vivo* for which further biokinetic modelling is needed.

There are different methods for carrying out a safety assessment and setting a quantitative point of departure that may be applied in risk management. A less-refined method could be to identify a threshold of toxicological concern (TTC) for a substance, based on extensive published toxicological data established for chemicals with similar structure and likelihood of toxicity (see also section 2.3). Taking a cautious approach, chemical structures have been

grouped into three broad categories, defined as low, moderate or high toxicity. Substances are conservatively assessed by comparing the appropriate TTC value with reliable human exposure data. If human exposure to a substance is below the TTC value, the likelihood of adverse effects is considered to be very low. The TTC values are used primarily for substances assumed to have low toxicity in order to avoid additional testing, e.g. certain cosmetics ingredients or food additives. The TTC value could be further informed by additional data from *in vitro* methods. This is suggested as a possible outcome in the level 2 case study on an AOP-based classification model to distinguish hepatotoxic and non-hepatotoxic substances.

Another safety assessment methodology is reading across, categorising or grouping. This is usually based on chemical structural relationships but could also be based on biological similarities (same mode-of-action). The level 3 'read-across' case study is further investigating how to carry out a robust read-across assessment incorporating new approach data (see section 3.5.1).

Traditional methods of complete risk assessment are based on exhaustive chemical data sets and are a costly and tedious exercise. There is a large potential to make chemical risk assessment more efficient by using alternative testing strategies based on *in vitro* and computational methods. Risk assessments could become both cheaper and faster, but also more relevant to human health, using human based models (e.g. cells of human origin in combination with human biokinetic models) rather than animal models. The work to develop such strategies has only just started and it will still take time until a complete substitution of traditional methods in risk assessment is possible. The level 3 *ab initio* case study is trying to achieve a full risk assessment based on alternative methods developed within **SEURAT-1**, in particular on the predictive toxicity case studies (see section 3.5.2). The *ab initio* case study will identify gaps which may assist in directing future research efforts towards the ultimate goal of complete replacement of *in vivo* repeated dose toxicity testing.

The starting point for any type of safety assessment is to search already existing data. The body of knowledge is increasing and becoming more accessible as databases and knowledge-bases are organised more intelligently, and information from many sources may be searched simultaneously in e-portals. When the already available information is insufficient, a first step would be to reinforce it with *in vitro* and *in silico* data. For chemicals with no or very little data, toxicity prediction strategies must be used. The case studies to prove the **SEURAT-1** concept are the first attempts to assess the safety of chemicals for repeated dose systemic toxicity, 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 (i.e., the SEURAT vision; *Whelan & Schwarz, 2011*).

The timing for completion of the different proof-of-concept levels is reported in the context of the **SEURAT-1** roadmap in section 4.11.1.

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3.3 Knowledge Level: Level 1 Proof-of-Concept Case Studies

3.3.1 Challenging the Predictive Power and Robustness of an Adverse Outcome Pathway Construct from Bile Salt Export Pump Inhibition to Cholestatic Injury

Mathieu Vinken, Vera Rogiers

Scope and Predictive Goal

The goal of this **SEURAT-1** proof-of-concept level 1 case study is to test the predictive power, robustness and reliability of an adverse outcome pathway (AOP) construct on drug-induced cholestasis. For this purpose, three selected liver-based *in vitro* models, including primary human hepatocytes (PHH), a liver hepatoma cell line (HepaRG) and a stem cell-based liver model (human skin-derived precursor differentiated to hepatic progenitor cells; hSKP-HPCs, see selected highlight of the DETECTIVE consortium in section 4.5.3), will be exposed to

bosentan (*Figure 3.4)*, a prototypical and potent cholestasis-inducing drug selected from the ToxBank Gold Compound list. The focus of this case study will be put on the detection of biomarkers. The anticipated intermediate steps and key events in the AOP will hereby be considered as benchmark biomarkers of drug-induced cholestastic injury. In fact, the relevance of each of the AOP information blocks, and thus the overall predictive value of the AOP, will be reinforced upon proper reproduction of the proposed intermediate steps and key events in the three experimental hepatic *in vitro* systems. At the same time, novel biomarkers will be identified by applying a number of '-omics'-based technologies, namely transcriptomics, epigenomics, proteomics and metabonomics. These new biomarkers may be potentially used as the basis for inclusion of as yet unidentified intermediate steps and key events in the AOP. Both the established and new biomarkers constitute the major deliverable of the DETECTIVE project, namely a set of biomarkers indicative of repeated dose hepatotoxicity. In turn, this deliverable will be directly fed into the other projects of the **SEURAT-1** Research Initiative, since it is one of the predefined building blocks required for the development of a strategy to aid in the replacement of current repeated dose systemic toxicity testing in animals.

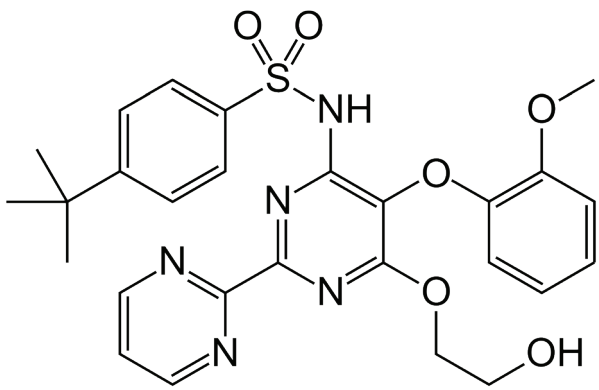

Figure 3.4 *Chemical structure of bosentan (4-tert-butyl-N-[6-(2-hydroxyethoxy)-5-(2-methoxyphenoxy)-2-(pyrimidin-2-yl)pyrimidin-4-yl]benzene-1-sulfonamide).*

Mechanistic Basis

An AOP construct for drug-induced cholestasis has been recently proposed by VUB (*Vinken et al., 2013*). In this AOP, *cis-inhibition* of the bile salt export pump (BSEP) is considered as the molecular initiating event. As a result of this event, toxic bile acids accumulate in hepatocytes or bile canaliculi. These bile salts trigger a direct deteriorative response and an adaptive response, both which form the basis for the intermediate steps and key events in the corresponding AOP. The deteriorative response is accompanied by the formation of

the mitochondrial permeability pore, which leads to mitochondrial impairment, inflammation, the production of reactive oxygen species and, ultimately, to the onset of cell death by both apoptotic and necrotic mechanisms. Because of the latter, cytosolic enzymes start to leak from hepatocytes and cholangiocytes and become measurable in the serum. The induction of the adaptive response is aimed at counteracting bile accumulation. Accordingly, a complex machinery of transcriptionally coordinated mechanisms involving nuclear receptors is activated by bile acids, which collectively decrease the uptake and increase the export of bile acids and bilirubin into and from hepatocytes, respectively. Simultaneously, detoxification of bile acids is enhanced, while their synthesis becomes downregulated. The increased effort of cholestatic hepatocytes to remove bilirubin causes bilirubinuria and hyperbilirubinemia. As a result, a yellowish pigmentation of the skin and the conjunctival membranes over the sclera becomes visible, known as jaundice. Furthermore, the elevated presence of bile acids in the serum is thought to account for the typical skin itching in cholestasis patients (*Vinken et al., 2013*).

Scientific Approach

The dual ETA and ETB endothelian receptor antagonist bosentan was selected as the positive control as it is known to cause cholestasis with the inhibition of the bile salt export pump as the postulated mechanism of action (*Fattinger et al., 2001; Morgan et al., 2010*). In a preliminary set of experiments, an appropriate concentration range of bosentan will be established by means of an MTT (cytotoxicity) test. In particular, and in view of establishing a concentration-effect relationship, three concentrations will be tested, namely an IC_{10} concentration based on the MTT test as well as 25% and 10% of this concentration. These concentrations will be established separately in each of the three liver-based *in vitro* models, namely PHH, HepaRG cells and hSKP-HPCs. In a subsequent series of experiments, the three liver-based *in vitro* models will be exposed to bosentan at the three established concentrations for 1 and 24 hours. To check for the reversibility of the effects, a subset of the cells treated with bosentan for 24 hours will be exposed to bosentan-free culture medium for another three days (wash-out period). At the three indicated times, samples will be taken for further testing. For establishing the overall competence of the *in vitro* models in generating markers of drug-induced cholestasis and to monitor the reproducibility of gene changes predicted in the AOP, microarray analysis will be performed. A reporter gene assay for FXR and PXR activation will be developed. For identifying new biomarkers of drug-induced cholestasis, the different '-omics'-based technologies, namely transcriptomics, proteomics, epigenomics and metabonomics, will be used. Quantification and concomitant statistical analysis of both the established and new biomarkers will allow the final selection of a set of *in vitro* read-outs of repeated dose cholestatic injury as well as the establishment of concentration-effects relationships.

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3.4 Methodological Level: Level 2 Proof-of-Concept Case Studies

3.4.1 Investigation of the Fibrotic Response Induced by Methotrexate and Acetaminophen in the *HeMiBio* Bioreactor

Leo A. van Grunsven, Sofia B. Leite, Mathieu Vinken, Pau Sancho-Bru, Yaakov Nahmias, Catherine Verfaillie

Liver fibrosis may result from a particular type of liver toxicity that can only be mimicked in complex hepatic *in vitro* models, i.e. consisting of at least hepatic stellate cells (HSCs) and hepatocytes that are preferably cultured in a 3D configuration. In *HeMiBio*, a hepatic microfluidic bioreactor is being constructed, *in casu* composed of genetically modified and/or stem cell-derived hepatocytes, HSCs and sinusoidal endothelial cells. Therefore, *HeMiBio* is among the few projects within the **SEURAT-1** Research Initiative that is able to screen chemicals for their liver fibrosis-inducing potential. In order to assess this property of the newly established *HeMiBio* bioreactor, liver fibrotic drugs, namely methotrexate and acetaminophen, will be tested in the *in vitro* setup and relevant biomarkers indicative of liver fibrosis will be monitored. The strategy is being mainly developed in the group of Leo van Grunsven (Vrije Universiteit Brussel, Brussels) in collaboration with Mathieu Vinken (Vrije Universiteit Brussel, Brussels), Pau Sancho-Bru (Institut d'Investigacions Biomediques August Pi i Sunyer, Barcelona), Yaakov Nahmias (The Hebrew Uuniversity of Jerusalem) and Catherine Verfaillie (Katholieke Universiteit Leuven).

The approach can be divided into three main phases. In the first phase, a cell culture setup will be optimised in order to have functional hepatocytes and HSCs for 21 days. This optimisation will be done in 3D Hepatocyte/HSC spheroid co-cultures of human hepatic cells kept in 96-well plates. These cells will be used once the human induced pluripotent stem cell-derived hepatocytes and HSCs become available, which allow monitoring using built-in reporter genes for several signalling and stress pathways.

Acetaminophen will be tested as it is a reference compound with a known outcome, i.e. hepatotoxicity and perhaps the ability to induce indirectly HSC activation. This serves as a quality control of the hepatocyte/HSC co-culture since it verifies effective metabolisation by hepatocytes (cells die from the accumulation of NAPQI, a CYP2E1-mediated metabolite of acetaminophen and this can indicate the quality of the cells after 21 days) and the potential of the HSCs to activate upon hepatocyte injury.

A second phase will consist of optimisation of the setup for fibrosis testing with methotrexate, a reported pro-fibrotic compound (*Kremer, 2004*), however with unclear pathway of hepatotoxicity and/or HSC activation. MTX will be first tested with the 'acetaminophen-optimised' setup, proceeding with the necessary adaptations upon testing. For practical reasons, both these phases will be performed in 96-well plates, while the third phase will consist of the adaptation into a bioreactor set-up under continuous perfusion, ideally with partial recirculation. In this set-up, the different sensors that have been embedded in the liver bioreactor will allow continuous and real-time monitoring of a number of parameters, including glucose consumption, oxygen consumption, lactate production, lactate dehydrogenase and alanine transaminase production.

Currently, the study is finalising phase 1; co-culture conditions have been optimised such that 3D HepaRG/HSC co-cultures retain hepatocyte functionalities and a non-activated state of HSC. Once exposed to different concentrations of acetaminophen (0-80mM) for 24 hours, a dose response toxicity can be observed as well as a dose-response increase of mRNA levels of several activation markers, which does not happen in 3D mono-cultured cells (HepaRG or HSCs). Work is ongoing to further extend the evaluation of fibrosis/activation state of HSC, namely optimisation of Loxl activity measurements and collagen protein detection in the culture supernatant.

Reference

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3.4.2 Evaluation of Valproic Acid Induced Steatosis in HepaRG Cells

Fozia Noor, Elmar Heinzle

In this case study the repeated-dose toxicity mediated via oxidation of fatty acids and leading to increased accumulation of lipids in vesicles will be investigated. Accumulation of lipids in the liver due to disrupted fatty acid and central metabolism results in 'fatty liver disease' or steatosis. Steatosis leads to steatohepatitis and often liver failure. Many drugs also cause steatosis upon repeated dose long-term exposure. The objective of this study is to obtain an '-omics' based mechanistic insight into steatosis. We have chosen valproic acid, which is known to cause hepatotoxicity via steatosis. Valproic acid and its metabolites are known to interfere with the β-oxidation of fatty acids and thus with energy metabolism (*Kesterson et al., 1984*). In addition, effects on nuclear receptors and gene expression have been reported (*Kiang et al., 2011*). Valproic acid is also known as an HDAC inhibitor. We will study valproic acid and its metabolites, their interference with fatty acid metabolism and their impact on the accumulation of lipids in vesicles. The resulting imbalance of lipid metabolism will be observed in 2D and 3D (spheroid) cultures of HepaRG cells. Repeated-dose long-term exposure to valproic acid with liver specific, as well as transcriptomic, epigenomic, and metabolomic measurements and flux analysis will be carried out. The exposed cell cultures will also be characterised using electron microscopy. All measurements provide time-series data outputs that will be used for setting up a mathematical prediction model of lipid accumulation. The goal is to extrapolate the effects observed in the HepaRG experimental model on the whole human liver using the computer model developed.

In the first phase, we have developed a long-term cultivation medium for HepaRG cells (*Klein et al., 2013*). This medium is also suitable for '-omics' endpoints (*Dahlmann, 2014*). We have already performed long-term concentration response studies on valproic acid in both 2D and 3D cultures (see section 4.7.4). On the basis of these experiments, concentrations that do not cause a decrease in cell viability of more than 20 % are chosen for further experiments. These concentrations range between 0.015-0.5 mM valproic acid. The *in vivo* concentration range of valproic acid is 0.3-0.5 mM. Therefore, we are carrying out our experiments at physiologically relevant concentrations. The exposure to valproic acid is carried out every second day upon change of the medium. We will also investigate the relevance of well-known toxicity pathways implied in steatosis.

The goal of this study is to develop an *in vitro* and an *in silico* model where steatosis could be investigated for the study on compounds with steatotic potential. After the investigation and calibrations of the system and the model with the **SEURAT-1** Gold Compound valproic acid, other chemicals can be used to investigate the prediction power of the model.

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3.4.3 Read-Across - Use of Biomarkers to Substantiate the Read Across Prediction

Sylvia Escher, Jan Hengstler

The lead question of this case study is whether biomarkers derived in the DETECTIVE project (see section 4.5) can predict *in vivo* data. A qualitative prediction is foreseen, which can be enriched by a quantitative approach if the qualitative approach proves to be feasible. From data-rich compounds (source compounds), a prediction of toxicity will be performed to data-poor compounds (target compounds), a methodology which is known as read-across. Here any mechanism of toxicity can be considered.

The unsupervised approach in the study can be described in the following steps:

➠ Identification of data-rich source compounds by analysis of gene array data. Gene array data from **SEURAT-1** partners and public sources (e.g. the TG GATE database) will be used to identify data-rich source compounds. Source compounds act by a common mechanism, or activate a common set of transcription factors. Within the first exploration, valproic acid was identified as a source compound that induces a set of enzymes involved in lipid metabolism. The same genes were altered in further compounds known to cause liver steatosis.

➠ Identification of source compounds having comprehensive *in vivo* repeated data. The toxicological profile of source compounds, including the effects and

targets observed in repeated dose toxicity studies of sub-acute to sub-chronic duration, will be analysed. The data-rich source compound should cause a specific adverse effect; in the case of valproic acid, liver steatosis. Candidate compounds will be applied to liver, kidney and heart test systems. Valproic acid has induced steatosis in different repeated dose toxicity studies.

➠ Structurally similar compounds to the data-rich substance should be identified, and if available, three types of 'similar' compounds will be distinguished: similar compounds with repeated dose toxicity data indicating a common mode-of-action; similar compounds with no further *in vivo* data (data-poor compounds); and similar compounds where repeated dose toxicity studies did not indicate the same mode-of-action.

For valproic acid, eight analogues matching to the three types of similar compounds were identified.

➠ Toxikokinetics: During the **SEURAT-1** Annual Meeting it was discussed that toxicokinetics are extremely valuable when assessing the feasibility of read-across for the selected compounds. Therefore, data on toxicokinetics will be searched within Tox21 data for all eight valproic acid analogues. Furthermore, COSMOS and the **SEURAT-1** Biokinetics Working Group (see section 4.11.6) will be contacted to see whether PBPK modelling for some of the analogues is feasible within the remaining project duration.

➠ Applicability of biomarkers: The identified compounds will be tested in the *in vitro* systems developed within the DETECTIVE project for the pre-defined biomarkers. Of particular interest is if the biomarker analysis would help to differentiate between compounds of different types as described above.

➠ Finally, the unexpected toxicity of analogues will be tested. At the **SEURAT-1** Annual Meeting it was decided that one aspect to be included in the case study is testing for 'unexpected' toxic events of all analogues. This point will be addressed within this case study to see which kind of assays are feasible and reasonable in this approach.

A supervised approach will also be applied. In this case, the compounds for which gene array data are available will be grouped according to mechanisms known from publications. An example may be drug-induced liver injury (DILI) versus non-DILI compounds. Based on these sets of positive and negative compounds, genes will be identified that best differentiate between both groups (candidate biomarkers). Next, the candidate biomarkers will be tested in a set of confirmation compounds; this is similar to the unsupervised approach.

3.4.4 Screening of Perturbed Toxicity Pathways by Transcriptomics Fingerprinting of Data Poor Substances

Agapios Sachinidis, Jan Hengstler

This proposed case study should complement the above-described case study on 'read-across' that will be based on data-rich scenarios allowing the generation of a hypothesis on the mode-of-action of the investigated compound and focused testing. In contrast to the 'read-across' case study, this case study concerns substances with limited '-omics' data or unclear 'dominant' modes-of-action. A 'transcriptomic fingerprinting' of the substance at physiologically relevant concentrations should allow the generation of a hypothesis on perturbed physiological pathways and possible associated hazards. The availability of such a hypothesis is needed for defining the next steps in a testing strategy, such as the selection of more sophisticated and predictive test systems that can provide the point-of-departure for further hazard characterisation. Additionally, the high sensitivity of the transcriptomics methodology also allows first predictions for the absence of adverse effects that are associated with 'general toxicity pathways' such as mitochondrial toxicity (prediction of negatives).

Data-poor scenarios are likely for many cosmetic ingredients because their mechanisms are not as well-defined as for pharmaceuticals. Therefore, the integration of a first screening early in the process of hazard identification/characterisation was also addressed at the **SEURAT-1** workshop 'The Development of Case Studies to Define Fit for Purpose Safety Risk Assessment of Repeated Dose Systemic Toxicity' (*White & Knight, 2013*).

The identification of pathway perturbation will be performed by assessing changes in the transcriptome of iPSC-derived cardiomyocytes and primary hepatocytes. The data acquisition will be performed using the Affymetrix transcriptomics platform and the data will be analysed statistically and interpreted by using standard tools, as previously described in peer reviewed publications by members of the case study team.

The innovative aspects in the case study include:

- The experimental design used to derive candidate biomarkers representing pathways relevant for delayed toxicities. Repeated dosing followed by a washout study allows the identification of pathways for which the perturbation is reversible or irreversible at physiological relevant concentrations;
- The qualification process of the selected biomarker, involving technologies that reflect different biological levels. This integrative approach allows the association of proposed biomarkers to a 'toxicity' pathway as presented by *Ankley et al.* (*2010*);
- Understanding of the relevance of mechanistic effects, such as mitochondrial toxicity for cosmetic ingredients (prevalence aspect);

➠ The investigation of chemically induced mitochondrial toxicity/oxidative stress in specific cell types to highlight similarities and differences with those induced by chronic conditions (as well as aging) common in aged individuals, as described in the literature. These will be used as benchmark for cellular and molecular changes, if any, induced by cosmetic ingredients;

➠ The identification of general and cardiomyocyte/hepatocyte-specific pathways.

The expected outcomes are:

➠ A concept of how to generate a hypothesis on the toxicity of data-poor compounds;

➠ An approach to map out the landscape of general and cell-specific toxicity pathways at transcriptome level relevant for selected cosmetic ingredients. The approach will allow the definition of the most prominent modulated pathways targeted by the selected cosmetic ingredients;

➠ An assessment of the relevance of well-known 'toxicity pathways' as specified by the **SEURAT-1** Gold Compound Working Group;

➠ A first understanding on the predictivity of the transcriptomic read-out for assessing negative compounds by a parallel evaluation of multiple toxicity pathways.

The repeated toxicity protocol and the transcriptomic approach will be applied to both cardiomyocyte and hepatocyte cellular systems. Initially, doxorubicin (cardiotoxicant) and bosentan (cholestasis inducer) will be applied to the cellular systems at clinical relevant concentrations; in the second phase other known hepatotoxic and cardiotoxic chemicals will be applied; and in a third phase cosmetics ingredients will be applied.

References

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3.4.5 Developing Chemotypes for Mitochondrial Toxicity

Mark Nelms, Kirk Arvidson, Steven Enoch, Elena Fioravanzo, Aleksandra Mostrag-Szlichtyng, Andrea Richarz, Christof Schwab, Lothar Terfloth, Chihae Yang, Mark Cronin

The aim of this case study is to develop an *in silico* profiler comprised of chemotypes to identify compounds with the ability to induce mitochondrial toxicity. A chemotype can be defined as a 'structural motif that can be augmented with physico-chemical properties and other descriptors in relation to toxicity' (*Yang et al., 2013*). *In silico* profilers can be utilised, for example, in the prioritisation of compounds to determine those chemicals that should be subjected to further testing. Interest has grown recently in understanding and developing toxicity pathways; one such approach that has been introduced is the adverse outcome pathway (AOP) paradigm. An AOP framework consists of a mechanistic connection between a regulatory relevant adverse outcome and its upstream molecular initiating event (MIE). The MIE is the initial interaction between the chemical and biological test system that initiates the progression of the pathway towards the adverse outcome.

Mitochondria are organelles present within most cells and organ systems within the body. Inhibition of normal mitochondrial function has been shown to result in organ-level toxicity. Recently, pharmaceutical companies have started performing mitochondrial toxicity assays as a routine part of the drug lead screening process (*Nadanaciva & Will, 2011*), as several drugs have been withdrawn from market due to organ-level toxicity induced by mitochondrial dysfunction. Within this case study, the MIE(s) of mitochondrial toxicity will be defined and placed in the context of (draft) AOPs. As the data for mitochondrial toxicity within the chemical selection literature are qualitative, an AOP will not be developed specifically for individual organs at this present time. Therefore, the chemotypes developed will be related to mitochondrial dysfunction in general.

Two separate data sets were utilised in the development of the chemotypes. The first data set consisted of 288 drug and drug-like compounds. This data set included a variety of chemical classes including phenothiazines, local anaesthetics and carbazoles; these were extracted from *Zhang et al. (2009)* with qualitative data for mitochondrial toxicity. The second data set was a group of 93 hair dye compounds comprising oral repeat dose toxicity data retrieved from Scientific Committee on Consumer Safety (SCCS) opinions (in collaboration with Professors Rogiers and Vinken, and Gamze Ates from Vrije Universiteit Brussel). For the first dataset, all structural analysis and subsequent chemotype development was performed using the freely available ChemoTyper software (available from https://chemotyper.org/). Structural similarity analysis of the second data set was undertaken using the Toxmatch software (v1.07). A detailed literature search was undertaken for those categories containing mitochondrial toxicants in order to elucidate mechanistic information related to the MIE(s) inducing mitochondrial dysfunction. Subsequently, information regarding the MIE(s) was used in the development of

thirteen chemotypes; ten relating to electron cycling, and three relating to proton cycling. The chemotypes developed will be implemented into KNIME or the ChemoTyper software.

A number of preliminary chemotypes have been defined across the two data sets for the inhibition of the electron transport chain and the uncoupling of oxidative phosphorylation via electron and proton cycling respectively. The chemotypes that have been developed will be used to identify any further compounds potentially capable of inducing mitochondrial toxicity. This information can then be used to refine the original thirteen chemotypes.

References

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3.4.6 Mode-of-Action-based Classification Model for Repeated Dose Liver Toxicity

Alfonso Lostia

The objective of this study is to develop a mode-of-action (MoA)-based classification model aimed at distinguishing between hepatotoxicants and non-hepatotoxicants. Hepatotoxicity is mainly related to three major liver adverse outcomes (AO) associated with repeated dose exposure: cholestasis, fibrosis and steatosis. The prediction goal is to minimise false negative predictions while ensuring an adequate discrimination between hepatotoxic and non-hepatotoxic chemicals. For chemicals identified as hepatotoxic, the identification of the specific liver AO (e.g. cholestasis, fibrosis and steatosis) is not within the scope of this case study. It is foreseen that the resulting classification model(s) will be useful for: (i) the hazard profiling of large chemical sets; (ii) priority setting (for further testing); and (iii) risk assessment by associating different thresholds of toxicological concern (TTC) with chemicals

that are predicted as positive and negative (positive chemicals will be associated with a lower TTC value; negative chemicals with a higher TTC value). In this case only liver toxicity is considered, but the idea is that the TTC value in the future could be related also to other toxicity endpoints of concern, and this case study would be a first step towards a better informed TTC approach.

Within the **SEURAT-1** Research Initiative, there is a good mechanistic understanding of the main MoAs underlying hepatotoxicity, which allows the design of MoA-based test systems. Particularly, from the analysis of the three MoAs for fibrosis, cholestasis and steatosis, three groups of key events can be identified and used to select the *in vitro* endpoints to be measured: (i) MoA-specific, (ii) common to multiple liver MoAs and (iii) describing general toxicity mechanisms and therefore associated potentially also to non-liver MoAs

A well-characterised *in vitro* liver system (HepaRG) will be exposed to 90 selected reference compounds and the knowledge of the three MoA key events will be used to select the *in vitro* endpoints to be measured. HepaRG cells are one of the preferred *in vitro* models for the liver since they possess a number of important hepatocytes characteristics (such as hepatocyte-like morphology, bile caniculus-like structure, competence for drug-metabolizing enzymes, transcripts of various nuclear receptors, liver-specific proteins, concomitant expression of hepatic influx and efflux transporters, and others).

High-throughput screening (using a 96-well plate format) will be employed to test the reference chemicals. The read-outs of the selected *in vitro* endpoints will be performed using High Content Screening (HCS) assays based mainly on automated imaging; other analytical techniques might also be used if necessary.

In order to increase the predictivity of the system and to facilitate *in vitro*-to-*in vivo* extrapolation, some toxicokinetic properties (such as bio-available/intracellular concentration and *in vitro* metabolic clearance) will be measured for some reference chemicals.

The analysis of the reference chemicals, comprising both known hepato- (positive controls) and non-hepato-toxicants (negative controls), will be used to build the classification model.

The positive reference chemicals must have clear and robust evidence that they cause hepatotoxicity based on *in vivo* data. We have compiled a list of pharmaceuticals known to cause hepatotoxicity based on human data in the literature. This list of chemicals was then expanded to include compounds from the **SEURAT-1** Gold Compound list (see section 4.11.3), the COSMOS DB, the ToxRef DB and the Hazard Evaluation Support System (HESS) database. The list also includes chemicals for which PBTK models are being developed within COSMOS to perform *in vitro*-to-*in vivo* extrapolation. Negative reference chemicals are also selected from the above-mentioned sources. For all reference compounds, the chemical diversity is explored (i.e. selecting structurally diverse positives and negatives).

Based on the *in vitro* endpoints, measured, specific criteria will be defined to discriminate

between positive and negative test responses in the HCS assay. These endpoints, along with structural descriptors (chemical alerts), will be used to build the classification models. The performance of the models will be assessed by investigating the percentage of false predictions given a predefined concordance. In other words, the desired concordance will be defined *a priori* when developing the classification models. The models will then be compared in terms of their ability to correctly identify negatives (negative predictivity). This is important in order to establish a higher TTC value for non-hepatotoxic chemicals.

3.5 Application Level: Level 3 Proof-of-Concept Case Studies

3.5.1 Read-Across Using SEURAT-1 Evidence

Derek Knight

Traditional read-across relies on the concept that chemical similarity leads to similar chemical and physical properties and thus similar toxicity. Such predictions can be confounded due to the underlying complex mechanisms of toxicity. The credibility of the scientific argument to support read-across may be supported by other information including test data. Information from *in vitro* molecular screening, '-omics' assays or computational models can be used to improve the robustness of the read-across case. In effect, using such 'new approach' **SEURAT-1** data as supporting evidence to improve the confidence in read-across based on similarity in chemical structure is equivalent to adding an examination of 'biological' similarity (as modelled by multiple short-term assays). The flexible 'conceptual framework' emerging from **SEURAT-1** can be used to design a specific workflow for the read-across case to combine the information from the **SEURAT-1** predictive tools in a rational manner. The type and degree of uncertainty in the predictions needs to be assessed and described accurately to ensure the prediction is 'fit for purpose'.

This case study on read-across is to demonstrate that the 'robustness' of 'read-across' of repeated-dose oral toxicity from a 'source' substance of known toxicology to 'target' substance(s) can be improved using SEURAT-type evidence. This is a realistic target within **SEURAT-1** that will be of practical regulatory use within the short term and hence reduce animal testing. It will also be a practical outcome from **SEURAT-1** that demonstrates a particular application of the approach of the 'conceptual framework', thus giving reassurance that broader application to *ab initio* prediction of toxicological properties will be feasible.

Good progress is being made in the read-across case study, with work done by the members of the Safety Assessment Working Group (SAWG), key members of the **SEURAT-1** projects and important input from the US EPA NCCT. The read-across case study was presented at the fourth **SEURAT-1** Annual Meeting in Barcelona, 5-6 February 2014, and at the joint meeting of the SAWG and DAWG (Data Analysis Working Group) the key issues of how to apply the 'conceptual framework' to read-across were considered as well as how to assess the impact of such **SEURAT-1** evidence for the adequacy of the read-across prediction. The European Commission's Joint Research Centre hosted an experts Workshop on 'The read-across case study for safety assessment contributing to the **SEURAT-1** Proof- of-Concept' in Ispra, Italy, 29-30 April 2014. The aims of the Workshop were to: (i) agree on read-across scenarios and select suitable chemicals pairs or categories; (ii) identify tests and data types to build up adequate read-across arguments; and (iii) advise on the integration of data using the logic of the workflow from the 'conceptual framework', including assessing and describing uncertainty. Further details about this workshop are reported in section 2.6.

3.5.2 *Ab initio* Case Study

Andrew White

This case study will show translation of findings and data from the integration of above-described relevant Level 2 case studies for a quantitative mechanistic safety assessment. The prediction goal is to determine a safe dose of an ingredient within a consumer use scenario. As the aim is to support the prediction of human health effects, the output will be benchmarked against published adverse data for the **SEURAT-1** Gold Compounds to assess the accuracy of predictions. Subsequently, the approach will be applied to a cosmetic-relevant ingredient to assess the ability to use this approach to bridge from pharmaceutical space to cosmetic space.

The case study outline follows the previously described flexible workflow (as outlined in section 4.11.8.1), covering the initial need to determine the critical MoA and then use higher level integrated models to provide a refined quantitative dose response estimate. These will be compared with published data for the **SEURAT-1** Gold Compounds to verify the predictive capacity of the system

A combination of approaches will be integrated to generate an hypothesis of the critical MoA for the compound including the use of: (i) Chemo-informatic approaches, alerts/chemical grouping; (ii) the distribution of the compound based on PBPK models; (iii) the hepatotoxcity classification model to assess liver relevance; and (iv) '-omics' data and HTS data from ToxCast assays, which will further aid in refining MoA and also attribute general or selective MoA for the compounds pathway.

An initial estimate of margin of safety could utilise the biological pathway altering dose (BPAD)

approach as described by *Judson et al. (2011)*. However, it is expected that differing models of data integration covering the range of biomarker information across the defined AOP will be explored. Additional refinement of the dose metrics by utilising free concentrations within the cell media and *in vitro*-to-*in vivo* extrapolation is expected to further strengthen the prediction. This, together with a prioritisation of those case studies that currently aim to provide a higher tier integration through integration of cellular responses incorporating cell crosstalk either within the *in vitro* system or via an *in silico* model, will be assessed to determine further improved assessment of a point of departure causally linked to adversity.

Variation and uncertainty from the predicted biokinetics will be included by use of population models and estimated exposure ranges from consumer habits data. However, further uncertainties, such as in population-based biodynamics not captured within an *in vitro* model, will need to be addressed as will extrapolation of the chronic dosing beyond that undertaken within the *in vitro* assays.

Relevant **SEURAT-1** Gold Compounds and a cosmetic relevant compound are selected based on the following outline criteria: (i) relevance and use for the defined AOPs; (ii) defined clinical dose that can be related to the AOPs; (iii) biokinetics models for compounds available or able to be developed; (iv) availability of screening data from '-omics' analysis and ToxCast data. Three compounds are suggested as a minimum to provide sufficient weight of evidence covering two liver AOPs. The compounds act as negative controls for non-related AOPs to show selectivity within the tissue, while a non-liver toxicant shows selectivity for non-liver related toxicities. A fourth compound from the cosmetic relevant chemical space is yet to be confirmed.

Current proposed **SEURAT-1** Gold Compounds are methotrexate, valproic acid and doxorubicin. A margin of exposure estimate will be generated for these compounds and the cosmetic-relevant ingredient from a predicted point of departure based on the *in vitro* assays and an estimated exposure based on consumer habit data and biokinetic modelling. The findings and approaches used within the case study, including identification of remaining capability gaps and uncertainties, will be written up and published as a proof-of-concept for *in vitro* based quantitative risk assessments.

Reference

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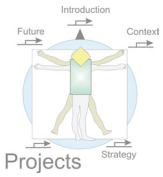

4 THE PROJECTS

"Millions saw the apple fall, but Newton was the one who asked why."

Bernard Baruch

4.1 Introduction

Tilman Gocht, Michael Schwarz

This chapter provides a comprehensive overview of the projects of the **SEURAT-1** Research Initiative and, thus, forms the backbone of the Annual Report. As the **SEURAT-1** proof-of-concept case studies are constantly developing, it starts with a summary of how the focus on hepatotoxic modes-of-action was established by **SEURAT-1**, finally leading to the formulation of the case studies outlined in chapter 3. Even though the focus at the cluster level is now shifting towards these case studies, it should be noted that the work programmes of **SEURAT-1** projects were formulated independently from them. Hence, there has also been much progress in the research projects outside of the **SEURAT-1** case studies, and this is reported in the subsequent sections with examples of elegant science and the achievements of the **SEURAT-1** projects, summarised in the respective 'highlight' sections of the project reports.

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 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 and third volume contained initial results from the research conducted within the **SEURAT-1** Research Initiative. This is continued in this fourth volume, which presents the research highlights from the third year in the context of 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 fourth year of the project; and (iv) future perspectives, describing possible next steps based on achieved and expected results from the various projects. An overview of the project Principal Investigators from each institution completes these sections.

Following the detailed project descriptions, a section summarising meeting reports at the project- and cluster-levels has been included. This section also contains extended abstracts from the awardees of the annual poster session organised at the fourth 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 over the lifespan of the **SEURAT-1** Research Initiative. The second volume of this Annual Report described the *modus operandi* of cross-cluster Working Groups as the central elements for facilitating the cooperation between projects and people. The third volume focused on the development of a **SEURAT-1** roadmap as a tool to monitor progress towards the achievement of the cluster-level objectives. This was extensively reported and has been briefly summarised and updated in this fourth volume, outlining how **SEURAT-1** as a whole is navigating towards achieving the final goal. This goal is to provide a blueprint for future implementation of mechanism-based integrated toxicity testing strategies into modern safety assessment approaches based on case studies demonstrating how far we can move away from the existing *in vivo* toxicity testing paradigm. Working Groups are playing a major role in these efforts and reports on activities and workshops conducted under the umbrella of the Working Groups complement this section. 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). All Working Groups include members from different research 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 needs identified 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 outreach activities completes this chapter. The central aspects here are: the organisation of the second **SEURAT-1** Summer School (held in June 2014); dissemination activities at conferences; the **SEURAT-1** public website; and the creation of a new leaflet with an enclosed USB stick containing all available volumes of the **SEURAT-1** Annual Report. Besides the Annual Report, these activities are the most important cluster-level tools to promote the dissemination of knowledge.

4.2 Focus on Hepatotoxic Modes-of-Action in SEURAT-1: Rationale and Strategy

Mathieu Vinken, Brigitte Landesmann, Vera Rogiers

4.2.1 Introduction

This chapter serves as a concise 'memorandum' that outlines the rationale for the major study focus on liver toxicity within the Mode-of-Action Working Group (MAWG) of the **SEURAT-1** Research Initiative. An overview is provided on the strategy adopted to implement (hepato)toxicological modes-of-action in the different projects. This approach consists of three steps:

- Step 1: identification of relevant toxicological modes-of-action and prioritisation of chemicals;
- Step 2: development of relevant adverse outcome pathway (AOP) constructs;
- Step 3: verification of the draft AOP constructs.

As such, these steps are consistent with the overall **SEURAT-1** strategy, implying proof-of-concept stratified into three distinct levels:

➠ Theoretical level: description of selected modes-of-action/AOPs to a sufficient extent so that they can be used as blueprints for system design;

➠ Systems level: demonstration of integrated systems for associating a chemical with a mode-of-action/AOP category and for predicting the points of departure of a pathway of toxicity;

➠ Application level: use of the information derived from predictive systems to support safety assessment and decision-making processes.

Steps 1 and 2 of the strategy described form the basis for the theoretical proof-of-concept level, while step 3 elaborates practically on both the systems and application aspects of the **SEURAT-1** vision. Further details are given in section 3.2.

4.2.2 Identification of Relevant Toxicological Modes-of-Action and Prioritisation of Chemicals

Context and Approach

The **SEURAT-1** Research Initiative intends to develop predictive toxicity strategies based on *in vitro* and *in silico* methods that contribute to the replacement of repeated dose toxicity testing using experimental animals. A key task at the start of this research initiative was the identification of target organs for *in vivo* systemic toxicity testing, as well as the characterisation of potential toxicological modes-of-action involved. This information was crucial for the selection of compounds to be tested in the **SEURAT-1** programme. Indeed, in the initial phase of this research initiative, *in vitro* systems and *in silico* models were developed using a set of chemicals with distinct toxicological properties, mainly pharmaceuticals. In a later phase, the developed tools should be challenged with a number of cosmetic ingredients. In order to establish a toxicological link between the chemicals used in both phases of the **SEURAT-1** programme, it was thus of utmost importance to find out which organs and potential types of toxicity could be of relevance to cosmetic ingredients. A primary resource that can be used for this purpose is the collection of safety assessment reports (or 'opinions') issued by the Scientific Committee on Consumer Safety (SCCS; *EU, 2008*), formerly called the Scientific Committee on Consumer Products (SCCP; *EU, 2004*) and the Scientific Committee on Cosmetic products and Non-Food Products intended for consumers (SCCNFP; *EU, 1997*). This scientific committee, active at the European level, addresses specific questions regarding the safety for human health of cosmetic ingredients and performs risk assessments of candidate cosmetic compounds to be included in the Annexes of the European Cosmetics Regulation (EC) No. 1223/2009. Clearly, these opinions contain a wealth of toxicological data. In an effort to improve access to this information, an electronic database was created by the Department

of Toxicology at the Vrije Universiteit Brussel-Belgium. This database includes scientific and publicly available content of the opinions issued since 2000 (*Pauwels et al., 2009; Rogiers & Pauwels, 2008*). In total, 253 opinions, involving 220 cosmetic substances, were screened (*Vinken et al., 2012*) and can be used as a starting point for selecting cosmetic ingredients as test chemicals in the **SEURAT-1** proof-of-concept case studies (see sections 3.3–3.5).

Outcome

It was found that the liver is the most prominent organ potentially affected during 90-day oral repeated dose exposure to cosmetic ingredients. This complies with the fact that the liver is the main site of xenobiotic biotransformation in the organism and thus represents a major target for toxicity. However, events such as changes in relative or absolute liver weight and hepatic hypertrophy do not reflect toxicity *per se*, as they can be a harmless and reversible manifestation of a hepatocellular adaptive response to the newly introduced compound (*Vinken et al., 2012*).

The liver's relevance as the organ most frequently affected by cosmetic ingredients was further assessed by listing all changes observed in histopathological and biochemical parameters that could point to hepatotoxicity (*Table 4.1*). Based on these combined observations, seven cosmetic ingredients were identified as plausible liver steatosis-triggering or cholestasis-inducing candidates (*Table 4.2; Vinken et al., 2012*).

Table 4.1 *Histopathological and biochemical parameters used to identify potential hepatotoxicity induced by cosmetic ingredients (Vinken et al., 2012).*

Hallmarks pointing to liver steatosis:

- Elevated blood concentrations of:
  - * Aspartate aminotransferase
  - * Cholesterol
  - * Triglycerides
- Fatty liver phenotype

Hallmarks pointing to cholestasis:

- Elevated blood concentrations of:
  - * Alkaline phosphatase
  - * Gamma-glutamyltransferase
  - * Bilirubin
- Hepatocellular necrosis

Table 4.2 *Cosmetic ingredients expected to induce liver steatosis and cholestasis (Vinken et al., 2012).*

Potential liver steatosis-inducing cosmetic ingredients:

- 3-methylamino-4-nitrophenoxyethanol
- Basic Brown 17
- HC Blue number 7
- Triclosan

Potential cholestasis-inducing cosmetic ingredients:

- 2-7-naphtalenediol
- Basic Red 51
- Triclosan

Similar screening exercises were performed by the Fraunhofer Institute in Hannover, Germany. These showed that liver fibrosis could also be identified as a potential manifestation of hepatotoxicity induced by cosmetic ingredients. Bearing this collective information in mind, the ToxBank consortium, in collaboration with the Gold Compound Working Group, selected a number of chemicals to be tested in the **SEURAT-1** programme, including those inducing liver fibrosis, steatosis and cholestasis (*Table 4.3*).

Table 4.3 *Chemicals known to induce liver fibrosis, steatosis and cholestasis selected by the* ***SEURAT-1*** *Gold Compound Working Group (ToxBank, 2014).*

Liver fibrosis-inducing chemicals:

- Allyl alcohol
- Methotrexate
- Carbon tetrachloride

Liver steatosis-inducing chemicals:

- Amiodarone
- Carbon tetrachloride
- Dirlotapide
- Rifampicin
- Tamoxifen
- TO901317
- Valproic acid

Cholestasis-inducing chemicals:

- Bosentan
- Chlorpromazine
- Tamoxifen

4.2.3 Development of Relevant Adverse Outcome Pathway Constructs

Context and Approach

In the last decade, predictive toxicology based upon mechanistic information has become a key aspect of human risk assessment. A major step in this direction came with the introduction of the mode-of-action concept, which relates to a series of key events along a biological pathway from initial chemical interactions to adverse outcomes (*OECD, 2012*). The mode-of-action concept was originally used by the US Environmental Protection Agency in the cancer field (*US EPA, 2005*), but seemed equally applicable for non-cancer endpoints (*Bogdanffy et al., 2001; Meek et al., 2003; Seed et al., 2005; Julien et al., 2009*). Another milestone was the well-known report published by the US National Academy of Science in 2007, outlining a vision on toxicology in the twenty-first century and placing toxicity pathways in the foreground (*NRC, 2007*). These toxicity pathways denote cellular pathways that can lead to adverse health effects when disturbed (*OECD, 2012*). Toxicity pathways align with AOPs, which have their roots in the area of ecotoxicology. An AOP is a conceptual construct that portrays existing knowledge concerning the linkage between a direct molecular initiating event (MIE) and an adverse outcome (AO) at a biological level relevant to risk assessment (*Ankley et al., 2010; OECD, 2012*). AOPs share a common structure, consisting of an MIE, a series of intermediate steps and key events, and an AO. Thus far, AOPs have been designed for a number of different human-relevant toxicological endpoints *(Vinken et al., 2013a; 2014*). In response to the increasing use of the AOP concept, the OECD has published a draft guidance document for the development and assessment of the completeness of AOPs (*OECD, 2012*).

Relying on the respective OECD guidelines on AOP development (*OECD, 2012*), AOPs from protein alkylation to liver fibrosis, from liver X receptor activation to liver steatosis and from bile salt export pump inhibition to cholestasis have been generated. Specifically, the two former AOPs (leading to fibrosis and steatosis, respectively) were established by the Systems Toxicology Unit of the Institute for Health and Consumer Protection at the Joint Research Center in Ispra, Italy (*Landesmann et al., 2012*), while the latter was introduced by the Department of Toxicology of the Vrije Universiteit Brussel-Belgium (*Vinken et al., 2013b*). In line with the OECD guidelines (*OECD, 2012*), the newly postulated AOPs have been evaluated with a

weight-of-evidence assessment using the Bradford Hill criteria (*Table 4.4*) and a confidence assessment using a set of key questions outlined in *Table 4.5*.

Table 4.4 *Bradford Hill criteria for AOP weight-of-evidence assessment (Hill, 1965; OECD, 2012).*

- Concordance of dose-response relationships.
- Temporal concordance among the key events and AO.
- Strength, consistency and specificity of association of the AO and the MIE.
- Biological plausibility, coherence and consistency of the experimental evidence.
- Alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP.
- Uncertainties, inconsistencies and data gaps.

Table 4.5 *Key questions for testing AOP confidence (OECD, 2012).*

- How well characterised is the AOP?
- How well are the initiating and other key events causally linked to the outcome?
- What are the limitations in the evidence in support of the AOP?
- Is the AOP specific to certain tissues, life stages or age classes?
- Are the initiating and key events expected to be conserved across taxa?

Adverse Outcome Pathway from Protein Alkylation to Liver Fibrosis

A crucial step in AOP development is the definition of the MIE, representing the interaction of a chemical with a biological system. In case of the liver fibrosis AOP, the MIE relates to hepatic protein alkylation or covalent liver protein binding. Different intermediate steps and/or key events at the cellular and tissue level have been defined including hepatocyte injury and cell death, activation of Kupffer cells, expression of transforming growth factor beta 1, activation of hepatic stellate cells, oxidative stress and chronic inflammation; collagen accumulation and changes in hepatic extracellular matrix composition (*Figure 4.1*) (*Landesmann et al., 2012*).

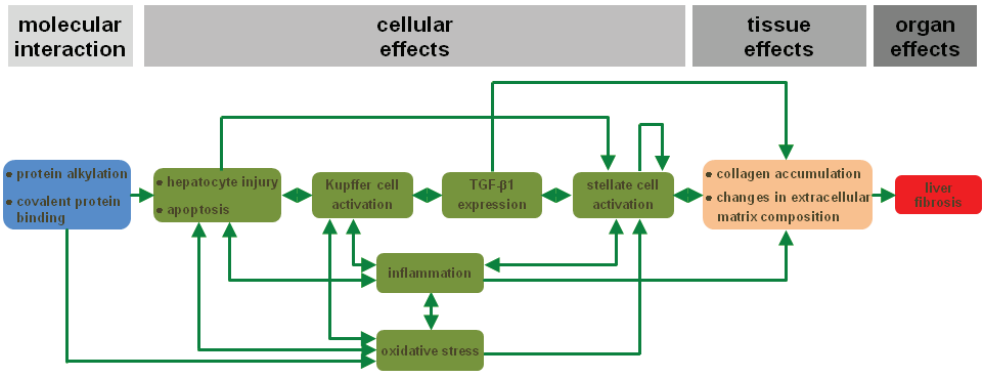

Figure 4.1 *AOP for chemical-induced liver fibrosis. The MIE (blue) is considered protein alkylation and covalent protein binding in the liver. This serves as a trigger to provoke hepatocyte injury, including apoptosis, which in turn activates Kupffer cells. As a result, the transforming growth factor beta 1 (TGF-β1) expression is induced, which is a key factor for stellate cell activation. The latter goes hand-in-hand with the occurrence of inflammation and oxidative stress. The different events at the cellular level (green) are interconnected in several ways. The overall end result is the accumulation of collagen and changes in the extracellular matrix composition in the liver (orange), which becomes clinically manifested as the AO, namely liver fibrosis (red) (adapted from Landesmann et al., 2012).*

Adverse Outcome Pathway from Liver X Receptor Activation to Liver Steatosis

The MIE in the liver steatosis AOP is the activation of the liver X receptor, which induces an array of intermediate effects, such as enhanced transcription of genes, encoding mediators of cholesterol and lipid metabolism. This leads to the increased influx of fatty acids from peripheral tissues into the liver and equally drives *de novo* synthesis of fatty acids. Consequently, triglycerides tend to accumulate in hepatocytes, which is considered a key event in this AOP. At the organelle level, hepatocellular lipid accumulation may provoke cytoplasm displacement, nucleus distortion, mitochondrial toxicity and endoplasmic reticulum stress. Altogether, these effects underlie the acquisition of the typical fatty liver cell phenotype, which in turn causes a clinically relevant increase in liver weight (*Figure 4.2*; *Landesmann et al., 2012*).

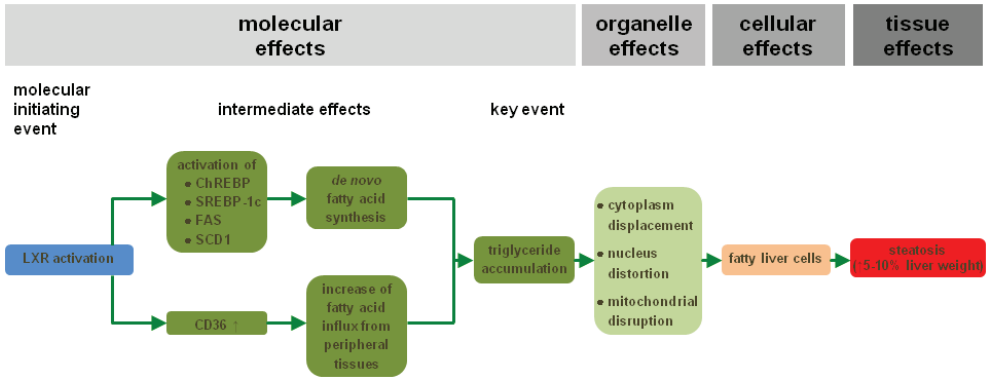

Figure 4.2 *AOP for chemical-induced liver steatosis. Activation of the liver X receptor (LXR), which is the MIE (blue), induces a number of transcriptional changes, including activation of the expression of carbohydrate response element binding protein (ChREBP), sterol response element binding protein 1c (SREBP-1c), fatty acid synthase (FAS) and stearoyl-coenzyme A desaturase 1 (SCD1). As a result, de novo synthesis of fatty acids is enhanced in the liver. At the same time, fatty acid translocase (CD36) production is upregulated, which mediates increased hepatic influx of fatty acids from peripheral tissues. All together, these intermediate steps drive accumulation of triglycerides, which is considered a key event (dark green). At the organelle level, this evokes cytoplasm displacement, distortion of the nucleus and mitochondrial disruption. This ultimately burgeons into the appearance of fatty liver cells (orange) and further into the clinical diagnosis of liver steatosis (red; adapted from Landesmann et al., 2012).*

Adverse Outcome Pathway from Bile Salt Export Pump Inhibition to Cholestasis

Cis-inhibition of the bile salt export pump is considered the MIE in the cholestasis AOP. As a result of this event, toxic bile acids accumulate into hepatocytes or bile canaliculi. These bile salts trigger a direct deteriorative and adaptive responses, both of which form the basis for the intermediate steps and key events in the corresponding AOP. The deteriorative response is accompanied by the formation of the mitochondrial permeability pore, which leads to mitochondrial impairment, inflammation, the production of reactive oxygen species and, ultimately, to the onset of cell death by both apoptotic and necrotic mechanisms. Because of the latter, cytosolic enzymes start to leak from hepatocytes and cholangiocytes and become measurable in the serum. The induction of the adaptive response is aimed at counteracting bile accumulation. Accordingly, bile acids activate a complex machinery of transcriptionally coordinated mechanisms involving nuclear receptors. Collectively, these mechanisms affect bile acids and bilirubin by decreasing their uptake into, and increasing their export from, hepatocytes. Simultaneously, detoxification of bile acids is enhanced, while their synthesis

becomes downregulated. The increased effort of cholestastic hepatocytes to remove bilirubin causes bilirubinuria and hyperbilirubinemia. As a result, a yellowish pigmentation of the skin and the conjunctival membranes over the sclera becomes visible, known as jaundice. Furthermore, the elevated presence of bile acids in the serum is thought to account for the typical skin itching in cholestasis patients (*Figure 4.3*; *Vinken et al., 2013b*).

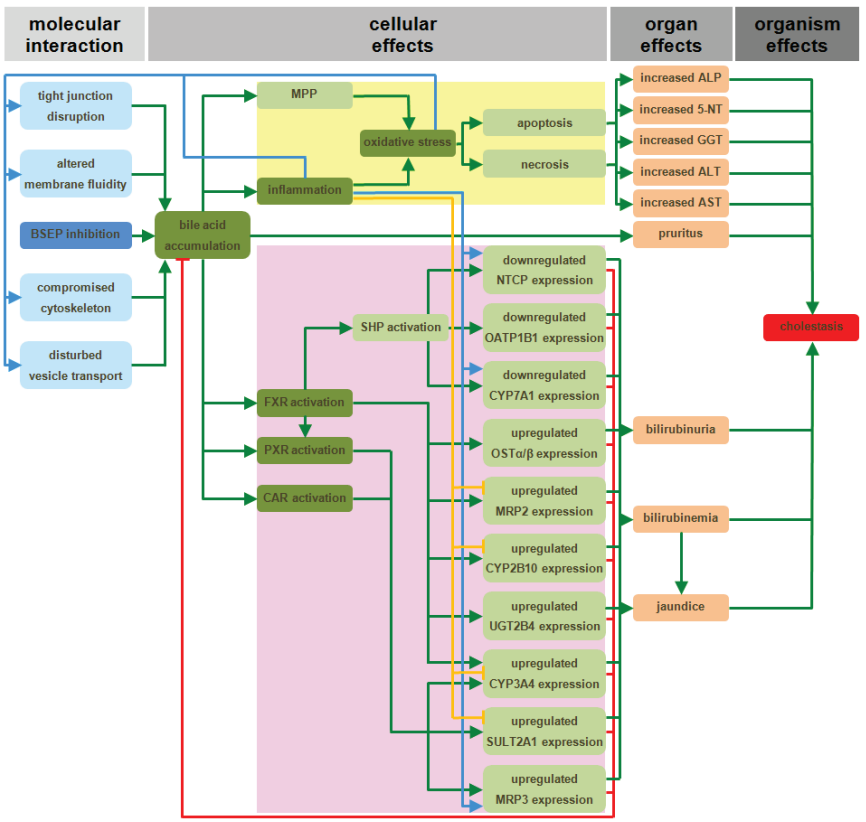

Figure 4.3 *AOP for drug-induced cholestasis. The response matrix between the MIE (dark blue) and AO (red), the inhibition of the bile salt export pump (BSEP) and cholestasis spans over the cellular and organ levels. Identified key events (dark green) include the accumulation of bile, the induction of oxidative stress and inflammation, and the activation of the nuclear receptors pregnane X receptor (PXR), farnesoid X receptor (FXR) and constitutive androstane receptor (CAR). Together with a number of intermediate steps, these key events drive both a deteriorative cellular response (yellow), which underlies directly caused cholestatic injury, and an adaptive cellular response (purple), which is aimed at counteracting the primary cholestatic insults. Direct inducing and inhibiting effects are indicated with green and red arrows, respectively. Secondary inducing and inhibiting effects of oxidative stress and/or inflammation are indicated with blue and orange arrows, respectively. (5'-NT, 5'-nucleotidase; ALP, alkaline phosphatase;*

ALT, alanine aminotransferase; AST, aspartate aminotransferase; CYP2B10/3A4/7A1, cytochrome P450 2B10/3A4/7A1; GGT, gamma-glutamyl transpeptidase; MPP, mitochondrial permeability pore; MRP2/3, multidrug resistance-associated protein 2/3; NTCP, sodium/taurocholate cotransporter; OATP1B1, organic anion transporter 1B1; OSTα/β organic solute transporter α/β; SHP, small heterodimeric partner; SULT2A1, dehydroepiandrosterone sulfotransferase; UGT2B4, uridine 5'-diphosphate-glucuronosyltransferase 2B4; Vinken et al., 2013b).

Verification of the Draft Adverse Outcome Pathway Constructs

Following establishment of the three AOPs on liver toxicity, the next step is to verify and challenge their actual relevance, robustness, reliability and predictive power. The **SEURAT-1** level 1 and level 2 proof-of-concept case studies are considered to be the ideal tools in this respect (see sections 3.3 and 3.4). Although not all of these case studies are specifically focused on liver toxicity, they can all contribute to the verification and further optimisation of the three AOPs. Each of the case studies will be scrutinised to maximise their value in this process.

4.2.4 Conclusions and Perspectives

As part of the proof-of-concept strategy, three AOPs on liver fibrosis, steatosis and cholestasis have been drafted (*Landesmann et al., 2012; Vinken et al., 2013b*). For the former and the latter, project applications have been submitted and approved by the OECD for the AOP development work programme and have been entered into the AOP knowledge base, an electronic system for the capture, management and sharing of AOP information. The three AOPs will now be verified and optimised in the context of the **SEURAT-1** case studies (see sections 3.3 and 3.4). In this respect, AOPs must be considered as open and flexible structures that should be continuously updated by entering established and newly generated data. Such iterative refinement exercises should ideally include the elaboration and quantification of the toxicodynamic relationships between neighboring events, as well as the specification of toxicokinetic conditions governing the activation of an AOP (*Vinken et al., 2013a and 2014*), all of which is foreseen in the presented **SEURAT-1** proof-of-concept case studies. Furthermore, it should be stressed that although hepatotoxicity and related AOPs are a main focus in **SEURAT-1**, a number of equally important ongoing efforts within the consortium, embedded in part of the submitted proof-of-concept case studies, address other organ-specific toxicities, including nephrotoxicity, neurotoxicity and cardiotoxicity. These AOPs and associated organ-specific *in vitro* and *in silico* testing approaches will be developed in parallel through the different **SEURAT-1** proof-of-concept levels.

AOPs can be used for a number of purposes, including the establishment of (quantitative) structure-activity relationships, the development of novel *in vitro* toxicity screening tests and the elaboration of prioritisation strategies (*Vinken et al., 2013a and 2014*). In the specific context of the **SEURAT-1** Research Initiative, the established AOPs and associated *in vitro* systems and *in silico* models will be tested for their applicability in generating predictive information to support safety assessment and decision-making processes.

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

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4.3.1 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*** programme 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.

4.3.2 Main Achievements and Challenges in the Third Year

These tasks of the first phase of the ***SCR&Tox*** programme were organised in order to provide all biological and technological resources needed for the second half of the program.

Essentially, human pluripotent stem cells were produced, protocols for differentiation into the five chosen lineages established, technologies for large scale production and banking set up and assessed, methodologies required for assessing responses of differentiated cells to toxicants identified and assayed in preliminary formats. These achievements have allowed us to undertake the second stage of the program aiming at demonstrating the value of pluripotent stem cells derivatives in toxicology studies at an industrial scale.

During several discussions, the ***SCR&Tox*** members have chosen the 'demonstrators' for the second half of the programme, *i.e.* the full assays that will be developed in order to meet the requirements of the industrial platforms and constraints. Two major lines of activities have been chosen to be the most relevant: the first will explore responses of keratinocytes and pluri-stratified epidermis and the second one will analyse neural cells. The approach is based on a genetically engineered iPS cell line expressing a reporter for activity of the Nrf2 transcription factor, as a marker of cell responses to oxidants. The methodologies for engineering cell lines have already been developed and validated, and different assay systems, meeting the requirements of the programmes, have been set-up. All teams involved are now moving on with the new tasks.

The ***SCR&Tox*** programme has been running very smoothly, with the exception of the difficulty in establishing some of the differentiation protocols. This had been envisaged in the original program and the provisional measure taken from the start was to pursue two paths for each lineage: one for a partially differentiated cell and other for a fully differentiated cell. The final outcome is at least one protocol available for drug toxicity testing for each of the lineages (both for the neural lineage, full differentiation for keratinocytes, and partial differentiation for the other three). Regarding the rest of the working programme, because of the lack of full differentiation for three lineages (that we have called 'late-stage'), the production of ready-to-use cells at the late stage has been withdrawn from the programme.

4.3.3 Selected Highlight: Human Pluripotent Stem Cell-Derived Neuronal Models for Toxicity Testing

Introduction and State of the Art

Neurotoxicity is one of the most challenging fields for the development of *in vitro* testing systems. In the last years, alternative *in vitro* testing strategies for chemical risk assessment have been designed, according to the current REACH legislation, to reduce the number of animal required for testing. However, to date *in vitro* assays for neurotoxicity have not been formally validated yet (*Bal-Price et al., 2010*). This is mostly due to the extreme complexity of the nervous system in which many different cell types are organised in a well orchestrated and functional network difficult to reproduce *in vitro*, but also to the lack of *in vitro* systems

and methods capable to fully cover some of the endpoints of the *in vivo* tests such as the neurobehavioral and neurocognitive aspects as well as the motor functionality. Nowadays, many cellular models are available for the nervous system, including primary cultures of fetal and adult neurons and glial cells, tumor-derived cell lines, hippocampal brain slices and neural progenitor cells, but all of this models suffer from considerable drawbacks, e.g. non-human origin, limited access or non-physiological transformed cell types (*Coecke et al., 2006*). Human pluripotent stem cells (hPSCs) are considered as a powerful tool for drug screening and the development of new *in vitro* testing strategies. Indeed, these cells can be indefinitely expanded and efficiently differentiated into neuronal derivatives, including different regionalised neuronal subtypes, glial cells and peripheral neurons (*Pistollato et al., 2014*). In this context, the European project ESNATS has taken the first step toward the design of developmental neurotoxicity tests based on the use of hPSCs, particularly developing battery of tests covering different aspects of neural teratogenicity (*Colleoni et al., 2010; 2012; Krug et al., 2013*). Many of these hPSCs-based models are very well characterised on the molecular basis, but to date there are few data indicating how they reflect the functionality of the *in vivo* central nervous system (CNS)/ peripheral nervous system (PNS) and clearly none of these systems can completely resemble the complex physiology of the entire nervous system. Consequently, the main problem in the development of novel test strategies relies on the fact that the mechanisms underlying neurotoxicity are too extensive to be covered with a single model and a small set of endpoints. Therefore, *in vitro* neurotoxicity tests should include different cellular models and multiple levels of evaluation, ranging from cytotoxicity and cell physiology to neuronal specific cell function endpoints. Moreover, the obtained data, in order to be considered reliable and predictive, should be compared across diverse *in vitro* models, extrapolated and further aligned to *in vivo* available data sets, in order to bridge the gap between *in vivo* and *in vitro* neurotoxicity.

Approach

In recent years, numerous protocols have been developed that enable the differentiation of hPSC into specific neuronal subtypes. As reported earlier, neural induction of hPSC can be efficiently achieved by the inhibition of transforming growth factor (TGF)-beta/activin/nodal (TGF-ß) and bone morphogenetic protein (BMP) signalling with SMAD signalling inhibitors (*Chambers et al., 2009; 2011*). During neural induction, hPSC undergo morphogenic events characterised by the formation of radially organised columnar neuroepithelial precursors appearing *in vitro* as 'neural rosettes'. Those neuroepithelial cells express early neuroectodermal markers such as Pax6 and Sox1 and are capable of differentiating into a broad range of region-specific neuronal and glial cell types in response to appropriate developmental cues (*Conti & Cattaneo, 2010*). The *in vitro* synthetic milieu is known to allow the expansion of such neuroepithelial stem cells in the presence of growth factors (EGF/FGF). However, prolonged exposure to growth

factors and cell culture ingredients like retinoic acid can deregulate the spatial identity and differentiation propensity of neural precursors, which results in a more restricted differentiation potential (*Elkabetz et al., 2008*). On the other hand, the derivation of stably proliferating neural stem cells from hPSCs further facilitates standardisation and circumvents the problem of batch-to-batch variations commonly encountered in 'run-through' protocols, which promotes terminal differentiation of hPSCs into somatic cell types without defined intermediate precursor stages (*Koch et al., 2009; Reinhardt et al., 2010; Li, et al., 2011; Falk et al., 2012*). Within the ***SCR&Tox*** project both, run through and stable intermediate differentiation protocols are available. For instance, ***SCR&Tox*** partner 'University Hospital Bonn' published the derivation of a long-term, self-renewing neuroepithelial stem cell population from hPSCs (lt-NES), which retains a constant neuro- and gliogenic potential even after long-term proliferation (>100 passages), and undergoes a pronounced restriction of its phenotypic and regional identity, which is mostly compatible with a ventral anterior hindbrain fate (*Koch et al., 2009; Falk et al., 2012*). In order to address the multiple aspects of neurotoxicity the availability of a variety of defined neuronal cultures is of utmost importance, since such well characterised *in vitro* systems are ideally suited to elucidate mechanisms of toxicity and to identify target cells of neurotoxicity.

Results

Differentiation of human embryonic stem cells (H9) and induced-pluripotent stem cells (IMR90) into post-mitotic neurons: Within the ***SCR&Tox*** project partner 'Joint Research Centre' developed an efficient protocol for the derivation of human neurons. The protocol is based on a run-through method starting from hPSC (either hESC or hiPSC), which were cultured on feeder cells (*Figure 4.4D*). The definition of neuronal differentiation protocols based on the use of human induced pluripotent stem cells (hiPSCs) would require a step-by-step comparison with the natural human embryonic stem cells (hESCs), in order to evaluate possible differences in differentiation potential between artificial and physiological hPSCs. Additionally, the comparison of the qPCR profiles would be useful as a quality reference standard, indicating the reproducibility of a differentiation protocol for a specific hPSC line prior to its implementation in large neurotoxicity screens.

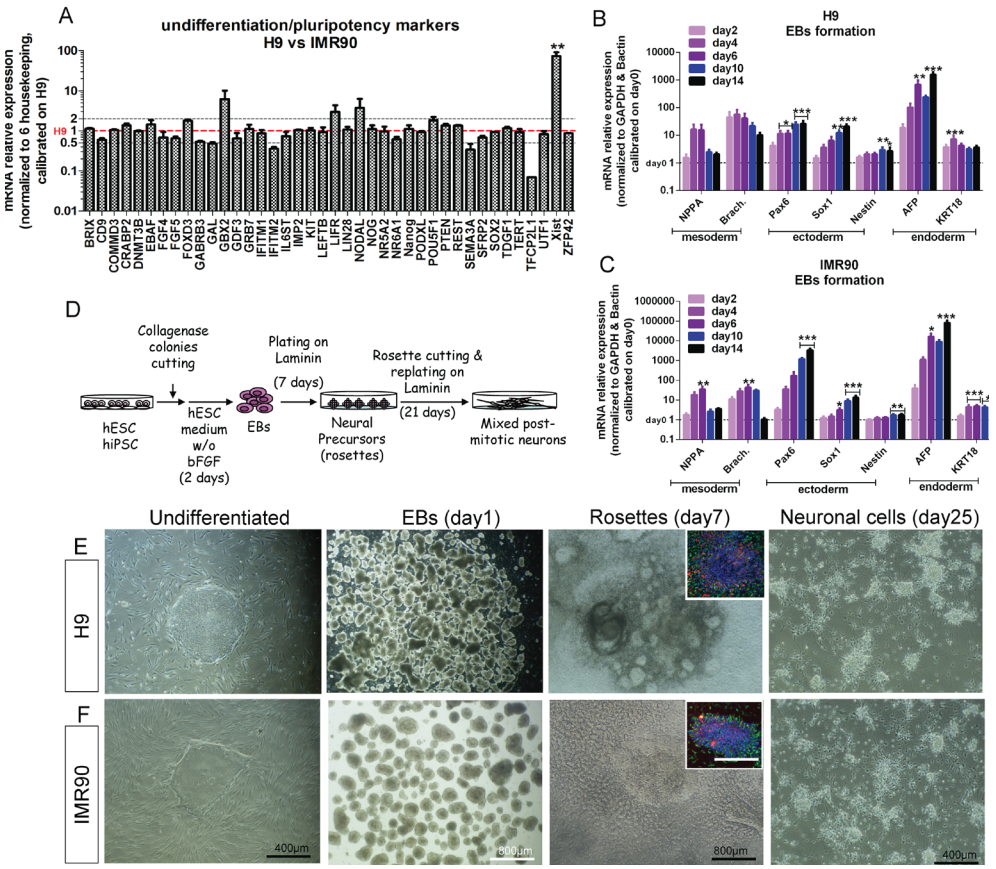

Figure 4.4 *Analyses of pluripotency related genes, EB formation and neuronal differentiation of PSCs. (A) Bar graph reporting qPCR analyses of pluripotency/undifferentiation related genes comparing undifferentiated H9 (dotted red line) with undifferentiated IMR90 cells, normalised to the 6 reference genes provided within the array (ACTB, RAF1, CTNNB1, GAPD, EEF1A1 and 18S) and then calibrated to the H9 (mean of 2 independent analyses ±SEM). (B, C) Bar graphs reporting qPCR analyses of indicated genes on H9- (B) and IMR90-derived EBs (C), normalised to β-actin and GAPDH and then calibrated to their own undifferentiated control (day 0, dotted line) (ΔΔCt method), mean of 5 independent analyses ±SEM. (D) Cartoon summarizing the differentiation protocol for PSC-derived post mitotic neurons. (E, F) (From the left) representative phase-bright images of undifferentiated colonies (H9p35 and IMR90p40), of EBs at day 1, of rosettes at day 7, with higher magnification insets showing nestin+ (green) and β-III-tubulin+ cells (red) (bar=100µm) and of neuronal cells at day 25.*

To this end, ***SCR&Tox*** partner 'Joint Research Centre' performed an extensive characterisation of the available hESC line (H9, from WiCell) and the hiPSCs (IMR90-hiPSCs, reprogrammed

in and provided by I-Stem). As a first step, undifferentiated H9 cells have been compared to undifferentiated IMR90 cells, by means of a customised Taqman human pluripotency array (Life Technologies), providing the simultaneous analysis of 34 genes related to stemness maintenance and pluripotency, 20 genes correlated to neuronal differentiation, 38 genes controlling the expression of other (i.e. non neuronal) differentiation related proteins and 6 reference genes. These data showed few differences comparing undifferentiated H9 vs IMR90, except for the expression of X-inactive specific transcript (Xist), a major effector of the X inactivation process, which resulted higher in IMR90 than in H9 (*Figure 4.4A*). Xist expression is variable in female undifferentiated hESCs (*Shen et al., 2008; Silva et al., 2008; Lengner et al., 2010*) and hiPSCs, which may reflect some variability in the epigenetic state and developmental potential of these cell types.

To assess pluripotency, the common approach based on 'spontaneous' embryoid body (EBs) formation was used and analysis of genes involved in the three germ layer formation was performed. Also in this case, H9 and IMR90-derived EBs resulted quite similar, with a significant increase of endoderm (AFP, KRT18), ectoderm (Nestin, Sox1 and Pax6) and mesoderm (NPPA and Brachyury-T) related gene expression in both cell models (*Figure 4.4B, C*). Comparing H9 and IMR90-iPSC expression, in particular a higher mRNA expression of Pax6 and AFP was recorded in IMR90-derived EBs than in H9-derived EBs, which might reflect some difference in differentiation propensity between the two lines.

Neural induction was achieved by EB formation and efficient neural rosette formation became visible upon re-plating of the EBs (*Figure 4.4D, E*).

Characterisation of PSC-derived neuronal cells: In order to characterise the different neuronal subtypes present in the H9 and IMR90-neuronal cultures, immunocytochemistry followed by High Content Imaging (HCI) was performed. VGlut1+ (i.e. Glutamatergic cells), GABA+ (i.e. gabaergic cells), TH+ (dopaminergic/noradrenergic neurons) and ISL1+ (i.e. motor neurons) could be detected in both cell models, whilst ChAT+ cells (i.e. cholinergic neurons) were not detected (*Figure 4.5A-C*). Additionally, qPCR analyses confirmed these data, indicating that especially dopaminergic, noradrenergic, glutamatergic, GABAergic and motor neuronal related genes were significantly up-regulated, together with some forebrain cholinergic genes, in both H9 and IMR90-derived neuronal cells vs. undifferentiated cells, with some differences in expression level between the two cell culture types (*Figure 4.5D*). In summary the obtained cultures represent a broad spectrum of neuronal subtypes, similar to the *in vivo* situation.

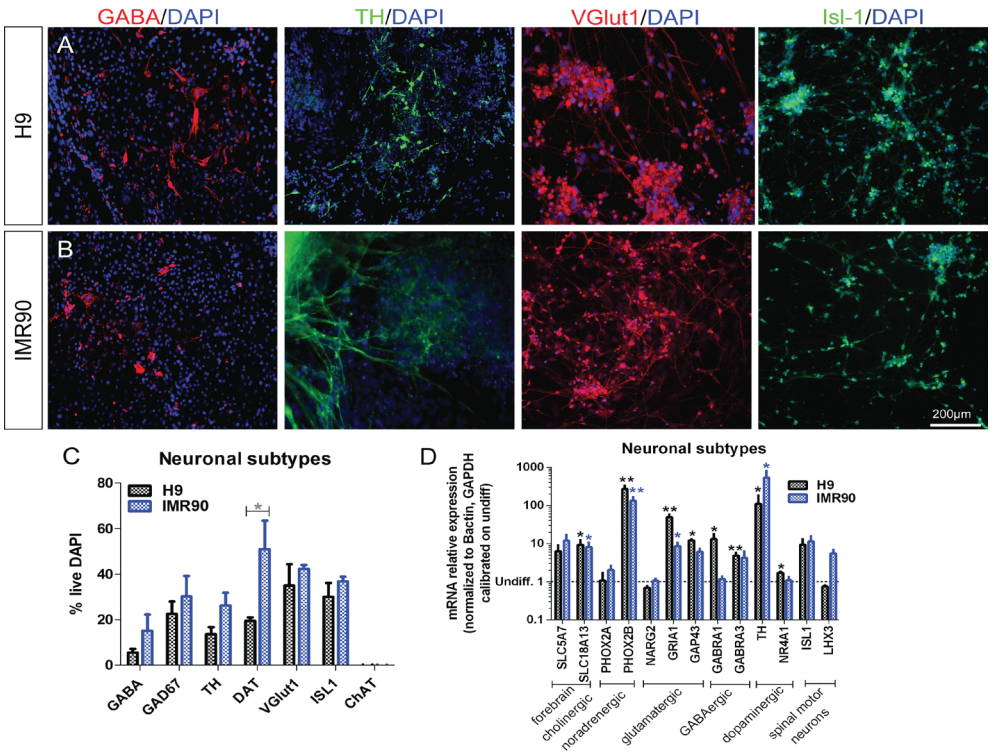

Figure 4.5 *H9 and IMR90 cells differentiate into GABAergic, glutamatergic, dopaminergic and motor neurons. (A, B) Representative immunocytochemical images of H9 and IMR90 derivatives stained with GABA (red), TH (green), VGlut1 (red) and Isl-1 (green) antibodies. (C) Bar graph reporting % of different cell sub-populations on total live DAPI+ cells (mean of 3 independent analyses ± SEM for both cell lines). (D) Bar graph reporting qPCR analyses of neuronal related genes normalised to β-actin and GAPDH and then calibrated to undifferentiated cells (dotted line for both cell cultures, ΔΔCt method) (mean of 3 independent analyses ±SEM for both cell lines).*

Analysis of signalling pathways expressed/activated in PSC neuronal derivatives: Defining which signalling pathways result to be activated upon neuronal differentiation would be relevant in order to select appropriate toxic compounds, possibly affecting these pathways, following the 'mode-of-action' framework approach established within the **SEURAT-1** Research Initiative. To this end, the ***SCR&Tox*** partner 'Joint Research Centre' assessed the expression of neuronal related genes, by using the customised TaqMan Human Protein Kinase Array (Life Technologies) (not shown) and the expression of neuronal related proteins, by using the reverse phase phosphoproteomic array (RPPA), in collaboration with the Department of Pediatrics of the University of Padua (Prof. Basso's group).

The RPPA analyses revealed that the pathways that resulted mostly up-regulated following differentiation are the cAMP response element-binding (CREB) related pathway, the PDK1/Akt/mTOR pathway, the SAPK/JNK and Notch1 pathway (*Figure 4.6*). These pathways were known to be relevant for neuronal differentiation and neurotoxicity and thus our results support the validity of our cellular model. Particularly, the CREB pathway is known to play critical roles in neuronal survival, dopaminergic neuron differentiation, precursor proliferation and neurite outgrowth and have also been shown to play a critical role in several toxicity insults and diseases (*Maizels et al., 2001; Schuh et al., 2002; Chalovich et al., 2006; Damodaran et al., 2009; Zuo et al., 2009; Xu et al., 2011*). A manuscript describing the molecular and cellular effects elicited by chemical-induced perturbation of the CREB signalling pathway is currently under revision (*Pistollato et al., 2014*).

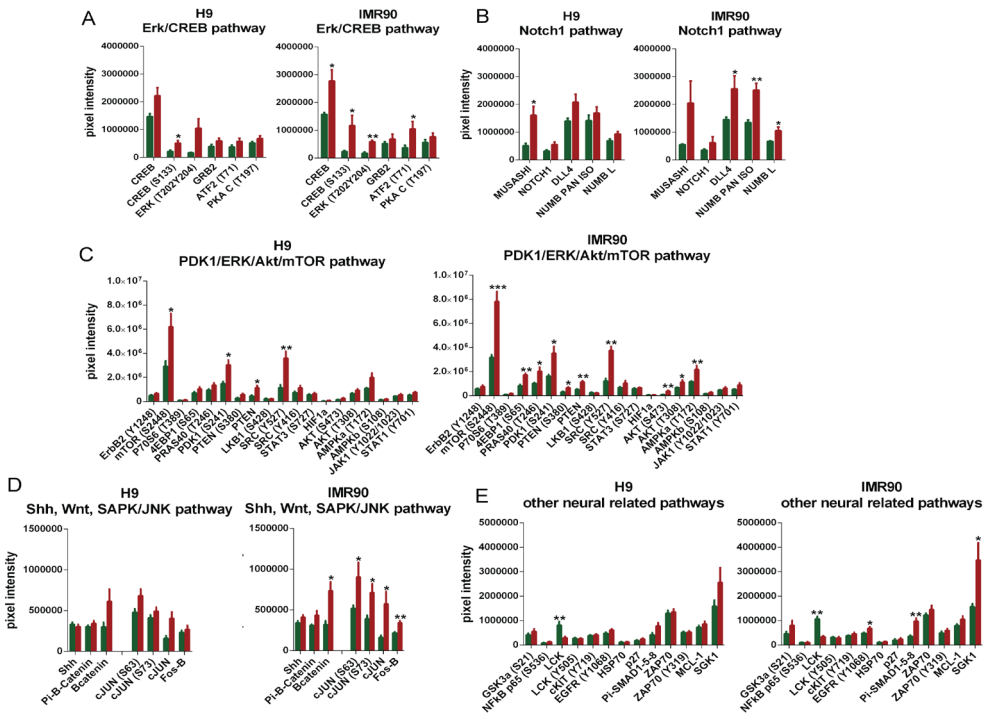

Figure 4.6 *H9 and IMR90 cells undergo upregulation/activation of neuronal related proteins. (A-E) Bar graphs reporting absolute protein quantifications using the Microvigene software, following RPPA analyses, comparing differentiated H9 and differentiated IMR90 (red bars) with their respective undifferentiated controls (green bars). Indicated proteins have been clustered together as indicated: Erk/CREB pathway (A), Notch1 pathway (B), PDK1/Erk/Akt/mTOR pathway (C), Shh, Wnt, SAPK/JNK pathways (D) and other neural related pathways (E) (Mean ± SEM of 4 independent analyses for both the cell models).*

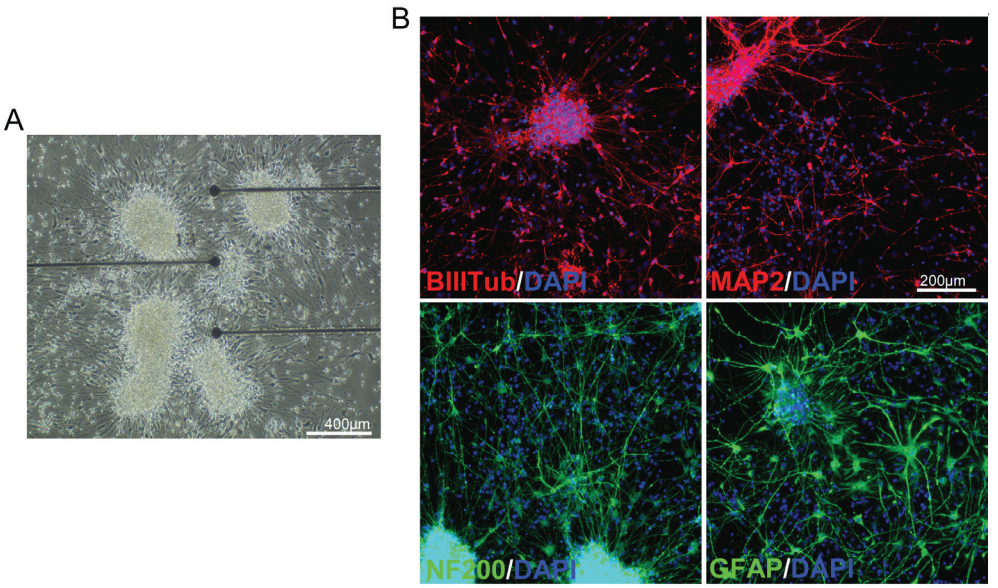

Figure 4.7 *Characterisation of human brain neurons (InnoProt). (A) Representative image of human brain neurons cultured on a MEA chip coated with laminin and (B) representative pictures of human brain neurons stained for the neuronal markers βIIITubulin (red), MAP2 (red), NF200 (green) and GFAP (green), and counterstained with DAPI (blue) for nuclei (10x magnification for all pictures).*

The MEA data revealed that both H9 and IMR90-derived neurons generated action potentials, with a mean firing rate (MFR, number of spikes/min) that was slightly, but not significantly, higher in IMR90 (mean of 58 spikes/ min) than in H9 cultures (mean of 38 spikes/ min). Opposed to what has been reported for other PSC-derived neuronal cells (*Heikkilä et al., 2009*) we could not observe spike bursts (i.e. trains of at least 2-5 action potentials/100 millisec, not shown). As expected, these recorded MFRs resulted slightly lower than the MFR recorded from the InnoProt human neuronal culture, being significantly lower than the one characteristic of rat cortical neurons, a classic animal neuronal model used in neurotoxicity assessments (*Figure 4.8A*).

Moreover, addition of tetrodotoxin (TTX, 1 μM), known to block action potentials by binding to the voltage-gated sodium channels, reversibly blocked the electrical activity in many of the recording MEA-channels, as shown in IMR90-neurons (Figure 5B) and as confirmed also in the human neuronal model (not shown).

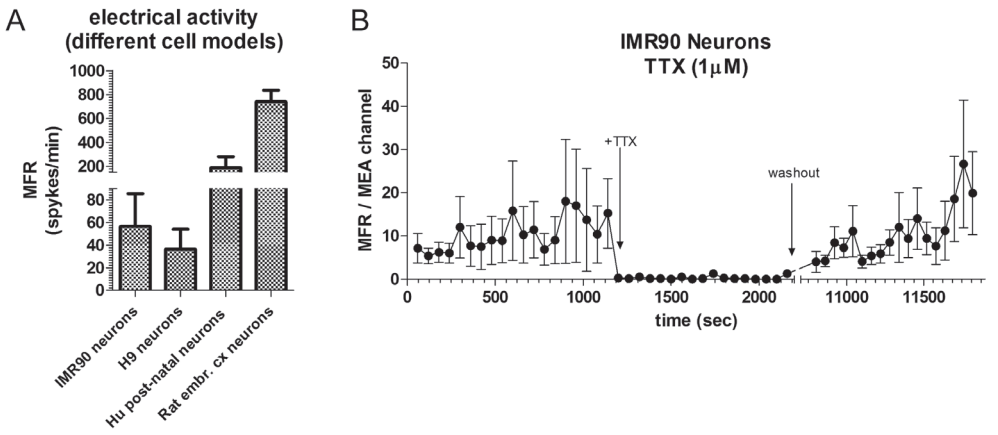

Figure 4.8 *Electrical activity analysis and comparison with possible benchmarking neuronal cell models. (A) Electrical activity analysis comparing different neuronal cell models: IMR90-neruons, H9-neurons, Human mature postnatal neurons (InnoProt) and Rat embryonic cortical neurons, as controls. (B) Graph reporting the effects of tetrodotoxin (TTX, 1μM) on TTX-sensitive IMR90-derived neurons (average of 4 representative MEA-channels). *p < 0.05, **p < 0.01, ***p < 0.001.*

As specifically requested by the ***SCR&Tox*** project, the focus should be on a repeated-dose toxicity study on terminally differentiated neurons. To fulfil this objective, the available neuronal cell culture models will be implemented in repeated-dose toxicity studies as outlined in *Figure 4.9*. Samples will be taken at day 1, 7 and 14 with the aim to detect early and long-term toxic effects. Multiple endpoints with different readouts will be considered to test the reliability of the system.

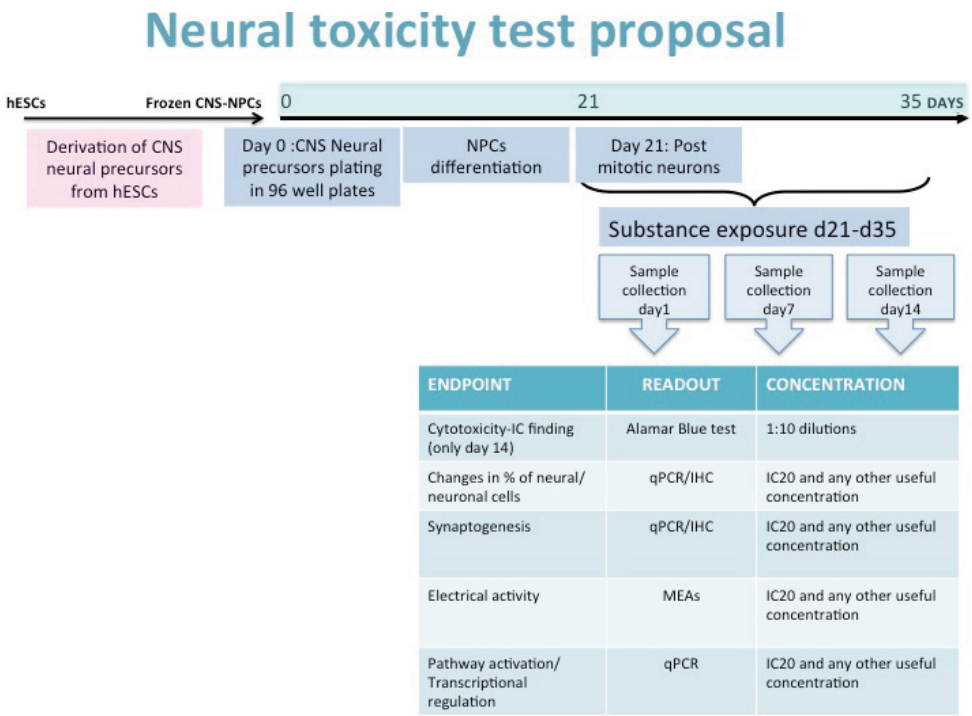

Figure 4.9 *Neural toxicity test proposal: an exposure of 14 days with samples collection at three different time points and assessment of multiple endpoints.*

4.3.4 Innovation

Human pluripotent stem cells can be triggered to differentiate into any cell type of the body, and hence offer the unique opportunity to develop a wide variety of *in vitro* human cell-based test systems. Therefore, hPSC are ideally suited to address the question if organ-specific toxicity can be predicted in an *in vitro* cellular model. The complexity of repeated-dose toxicity can involve a number of different target organs and even when adversities *in vivo* are various, there is strong evidence that the different manifestations can be triggered by the perturbation of identical pathways. The ***SCR&Tox*** consortium has taken on the task to determine the relevance of the Nuclear factor (erythroid-derived 2)-like 2 (Nrf-2) toxicity pathway across a sub set of tissue types (e.g. keratinocytes, neurons and cardiomyocytes), which are now available at sufficient quantities and quality among the ***SCR&Tox*** partners. For neuronal tissue one of the main challenges is the fact that the cellular diversity within the brain is too extensive to be covered with a single cellular model and a small set of endpoints. As a prerequisite, during the ***SCR&Tox*** project differentiation protocols for the derivation of a variety of neuronal subtypes from hPSC have been established, including the one from ***SCR&Tox*** partner 'Joint Research Centre' that is presented here in more detail.

It is becoming clear that different chemical entities can cause oxidative, genotoxic and proteotoxic stress, which induce cellular responses in an effort to restore homoeostasis. One of the primary involved response pathways is the Nrf-2 pathway (*Jennings et al., 2013*). The Nrf-2 pathway is a master regulator of the ARE-driven cellular defence system against oxidative stress and several studies have shown that Nrf-2 protects many cell types and organ systems from many toxic insults and disease pathogenesis (*Lee et al., 2005*). More particularly, upon activation Nrf-2 binds to ARE sites in the promoter regions of many detoxification genes, leading to up-regulation of downstream targets that support detoxification processes and antioxidant potential (*Petri et al., 2012; Ma, 2013*). Nrf-2-dependent transcriptional activation has been shown to be protective against neural toxicants (*Table 4.6*), which are also implicated in neurodegeneration and major neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (*Picklo et al., 2002; Lee et al., 2003; Barbeito et al., 2004; Calkins et al., 2005; Lee et al., 2005; Ma, 2013*).

Table 4.6 *Chemicals relevant for studying Nrf-2 pathway for neurotoxicity evaluation.*

Chemicals
Tert-butyl hydroperoxide
1-methyl-4-phenylpyridinium (MMP)
Malonate (mitochondrial complex II inhibitor)
Paraquate
3-nitropropionic (3-NP) (mitochondrial complex II inhibitor)
6-hydroxydopamine
1-methyl-2-phenyl-1,2,3,6-tetrahydropyridine (MPTP or MPP+)
Synthetic triterpenoids (TP)
Inomycin or dtBHQ (increased calcium influx inducing oxidative stress)
Manganese
Methyl mercury

In order to decipher organ-specific toxicity on the basis of the available neuronal cellular models, Nrf-2 activation will be assessed via ARE-Luciferase reporter gene activation and gene expression analysis of Nrf-2 downstream target genes, which are implicated in increased cellular energetics, redox potential, inhibition of the neurotransmitter signalling and oxidative stress. To explore the relevance and predictivity of Nrf-2 activation upon compound treatment, correlative studies employing neuronal cell-type specific endpoint analysis will be performed.

Functional endpoint analysis will include the reported bioelectronic monitoring system for stem cell-derived neuronal networks (*Robitzki et al., 2013*). In conclusion, the ***SCR&Tox*** project provides the cellular and technological resources needed to assay toxicity pathways *in vitro* and to demonstrate that these resources can be reliably and robustly implemented at the required scale.

4.3.5 Cross-Cluster Cooperation

Interactions with other networks of the **SEURAT-1** Research Initiative are very active, specifically with DETECTIVE, with which ***SCR&Tox*** will share the approach of focusing on the Nrf-2 pathway. COACH members have been very actively contributing to the set-up of that project as well, by participating to meetings of the ***SCR&Tox*** network dedicated to that issue that have taken place over the past year. It is important to notice that participation of the Scientific Advisory Board on ***SCR&Tox*** meetings has been extremely active and positive. Ian Cotgreave, the chair of **SEURAT-1** Scientific Expert Panel, has also actively participated to the meeting that made the switch between the first and the second step of the programme.

Members of ***SCR&Tox*** have had a number of interactions with members of other **SEURAT-1** consortia: in June 2012, a joint meeting of *HeMiBio*, ***SCR&Tox***, NOTOX and DETECTIVE was held to discuss bioreactors, cells and genetic engineering of cells. There was subsequently a joint teleconference organised in January 2014 by ***SCR&Tox*** with DETECTIVE to discuss the reporter cell models each consortium had or were planning on using and contact was made with members of both *HeMiBio* and DETECTIVE specifically regarding the use of Nrf-2 reporter cell lines in each of the consortia. DETECTIVE has expressed an interest in obtaining the iPS Nrf-2 reporter cell line from ***SCR&Tox*** once available to use in their work plans for investigating renal perturbations of the pathway.

The fact that some ***SCR&Tox*** partners participate in other consortia from the **SEURAT-1** Research Initiative fosters collaboration between different projects. ***SCR&Tox*** partner 'Karolinska Institutet' is involved in NOTOX and partner 'Joint Research Centre' is involved in the DETECTIVE consortium. The latter was very important in organising a first joint session on repeated dose exposure protocols in heart models with members from DETECTIVE and ***SCR&Tox***. Finally, the ***SCR&Tox*** partner 'National Institute for Biological Standards and Control – Health Protection Agency' is also a member in ToxBank.

Finally, ***SCR&Tox*** is leading the **SEURAT-1** cross-cluster Stem Cells Working Group (SCWG).

4.3.6 Expected Progress within the Fourth Year

The assay development will be dedicated to the neurotoxicity programme using the engineered

iPS cell lines characterised by the New Generation Sequencing technologies. Neurotoxicity will be evaluated on the fully differentiated neurons using the different techniques obtained during the first phase of the project. The specific goal is to develop an assay for oxidative stress – a toxic mechanism that is frequently observed in neurons – based upon the use of Nrf-2 translocation to the nucleus and the over-expression of its known molecular target genes. This assay, once validated experimentally, will be developed in two additional directions. First, technology transfer will be organised toward the platforms of the industry partners of the network. Second, all documentations needed for submission of the assay to the regulatory authorities will be prepared. Providing the assay performs as planned, we plan to submit an application at the end of the ***SCR&Tox*** project.

In parallel, we start immediately translation of the technologies developed within the ***SCR&Tox*** project towards industrial platform using the epidermis model. This will also make use of the cell lines engineered and produced within the project and the deep sequencing activity. Assays for skin toxicity using the Nrf-2 construct as a marker for oxidative stress have already been developed and validated by regulatory authorities (the so-called 'keratinosens test'). There are major potential advantages of developing our tests with iPS-derived cells, in particular the ability of those cells to form a three-dimensional epidermal structure, which cannot be obtained with current concurrent cell lines used in the keratinosens test. A number of products, in particular in the cosmetic domain, cannot be fully evaluated in 2D format. The programme on skin toxicity will first aim at reproducing strictly, in 2D, the keratinosens test. In this way we will validate the cells obtained from iPS as a platform for toxicology demonstrating that they perform as good as the accepted model systems. Subsequently, pluri-stratified epidermis will be allowed to grow in multi-well plate format, and skin toxicity will be evaluated in 3D. This will demonstrate the added value of our cell models and provide a strong basis for proposing the new cell system to the regulatory bodies and industry.

4.3.7 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 subacute 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 4.10*.

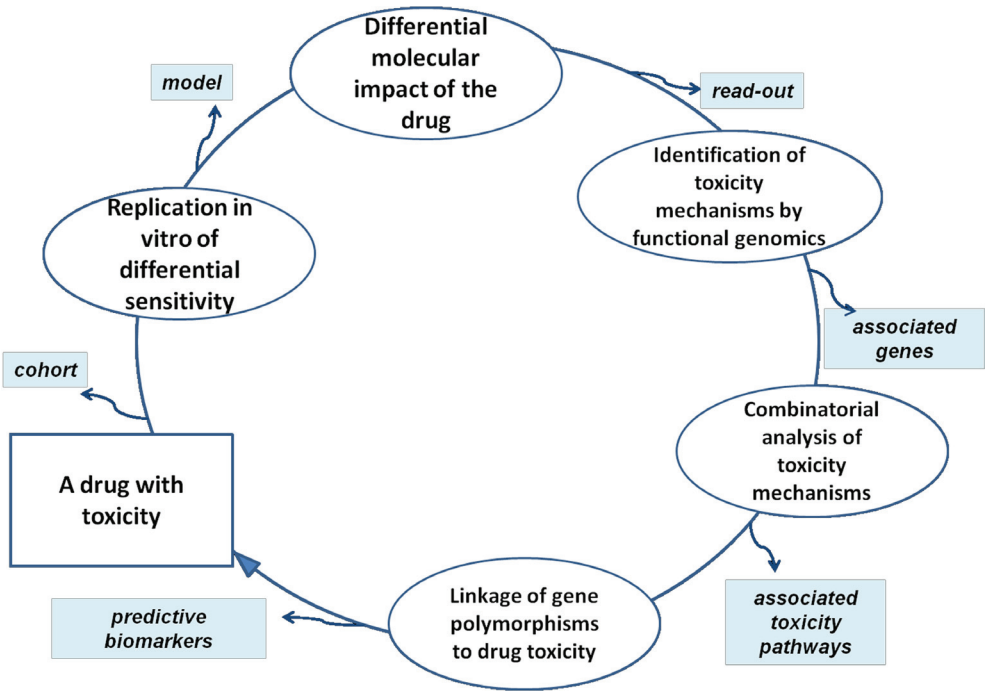

Figure 4.10 *Outline of an innovative approach for harnessing pluripotent stem cells for toxicology.*

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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4.4 HeMiBio: Hepatic Microfluidic Bioreactor

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4.4.1 Introduction and Objectives

Refinement, reduction and replacement of animal usage in toxicity tests (the 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 aimed at implementing the 3Rs principle in toxicity testing have been funded by the European Commission, 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 humans 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 functions, 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 the 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.

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. Therefore, we propose to isolate the cellular components from differentiated pluripotent cells. Pluripotent cells are normally derived from blastocysts, as embryonic stem cells (ESCs). Alternatively, they can be created from mature terminally differentiated cells by the introduction of pluripotency genes, that 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 LSECs and HSC features, which will be used to generate the liver-simulating device. We also believe that the 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.

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) the *matrix* whereupon cells are maintained, *oxygenation*, and *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; and (iv) the system needs to be built such that *repeated on-line 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 4.11*) may not be required, the 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, (C) and the presence of monocytes/Kupffer cells will be required to fully assess drug toxicity.

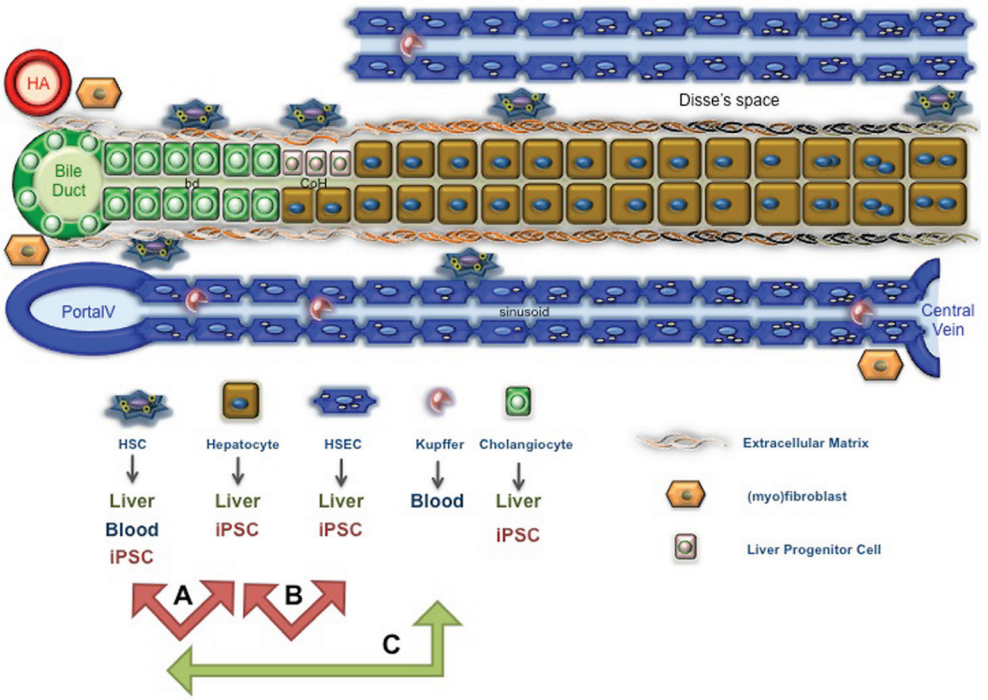

Figure 4.11 *Schematic representation of a liver sinusoid (adapted from: Dollé et al., 2010).*

To create 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* that 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. This 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.

4.4.2 Main Achievements and Challenges in the Third Year

During the last year, extensive focus was on: (i) further optimisation of the cell-culturing methods and subsequent characterisation of hepatic stellate cells and liver sinusoidal endothelial cells; (ii) the differentiation of pluripotent stem cells towards hepatocytes, hepatic stellate cells and liver sinusoidal endothelial cells; (iii) testing of the **SEURAT-1** standard reference compounds on hepatocytes treated with the UpCyte® technology; (iv) the development of molecular-engineered pluripotent stem cells; (v) the improvement and generation of microsensors; and (vi) the validation of the flow-over bioreactor and initial testing of the flow-through bioreactor. Furthermore, ***HeMiBio*** contributed actively to the formulation of **SEURAT-1** case studies.

Optimisation of the Cell-Culturing Methods and Characterisation of Hepatic Stellate Cells and Liver Sinusoidal Endothelial Cells

As described below (see section 4.4.3), we have completed the molecular and epigenetic profiling of primary liver derived hepatic stellate cells. These data were presented at the fourth **SEURAT-1** Annual Meeting in February 2014 in Barcelona, Spain, as well as at international conferences. A publication is being prepared that will describe these findings.

We have further evaluated means of maintaining hepatic stellate cells quiescent, by modifying culture media and conditions. In addition, we have developed standard operating procedures enabling the generation of co-cultures between the cell line HepaRG and primary hepatic stellate cells, wherein hepatic stellate cells remain quiescent but can be activated upon fibrogenic stimulation.

For liver sinusoidal endothelial cells, less progress has been made to maintain their non-activated phenotype in culture. However, as for the hepatic stellate cells, we have now completed studies aimed at understanding the molecular make-up of liver sinusoidal endothelial cells; this will aid in developing methods to maintain these cells *in vitro*.

Differentiation of Pluripotent Stem Cells Towards Hepatocytes, Hepatic Stellate Cells and Liver Sinusoidal Endothelial Cells

Two teams from the ***HeMiBio*** consortium have developed methods to support hepatocyte-like cell generation from pluripotent stem cells. Such cells have the properties of hepatocytes, even if at 1-10% of primary uncultured hepatocytes. Nevertheless, preliminary studies have shown that this hepatocyte progeny may be suitable for studying toxic effects of the **SEURAT-1** standard reference compounds, identified by the Gold Compound Working Group (see section 4.11.3).

Testing of the SEURAT-1 Standard Reference Compounds Hepatocytes treated with the UpCyte® Technology

An initial evaluation of the toxic effects of different compounds in hepatocytes treated with the UpCyte® technology was published during the second year of the project (*Burkard et al., 2012*). During the third year a more systematic evaluation of the suitability of hepatocytes treated with the UpCyte® technology for drug toxicity assessment, specifically focussing on the **SEURAT-1** standard reference compounds, was completed and a manuscript was submitted. Further validation of the cells for drug toxicity screening in additional labs is being initiated.

Further Development of Molecular Engineered Pluripotent Stem Cells

During the past year, we have generated donor plasmids for lineage tracing, inducible overexpression of transcription factors and inclusion of toxicology readout cassettes. These were exchanged with flipase in the 'safe harbour' AAVS1 location previously engineered in pluripotent stem cells by homologous recombination. A manuscript was submitted describing the possible applications of this technology.

Improvement and Generation of Microsensors

Development and characterisation of sensors for the monitoring of cell culture death and function has continued. Long-term stability of one month for potassium, ammonium and ALT sensors has been achieved. Urea sensors functional for a short period (one day) are available. Work is in progress to improve the mechanical stability, and thus the lifetime of these sensors.

Bioreactor Developments.

As discussed below (see section 4.4.3), the flow-over bioreactor has been validated for its suitability to sustain hepatocytes for several weeks (manuscript submitted). Several iterations of the flow-through bioreactor have been generated and are ready for testing.

Contributions to SEURAT-1 Case studies

HeMiBio has initiated the level 2 case study 'Investigation of the fibrotic response induced by methotrexate and acetaminophen in the ***HeMiBio*** liver bioreactor'. Being the only **SEURAT-1** project working with different hepatic cell types, including hepatic stellate cells (HSCs), ***HeMiBio*** studied the activation of HSCs to test fibrosis. HSCs are the leading cells in a fibrotic response that upon injury can trans-differentiate into myofibroblast cells, release lipid droplets, and proliferate and increase their ECM production. However, the injury is often not directed to HSCs but to the surrounding cells, mainly hepatocytes. For this reason, this case study is based on compound testing in hepatocyte/HSC co-cultures. The implementation of the study was divided into three parts: (i) development, optimisation and testing of culture conditions that allow the efficient culture of functional hepatocytes and HSCs for three weeks; (ii) optimisation of the setup for fibrosis testing with methotrexate; and (iii) translation of the developed model into a bioreactor.

The first phase is close to finalisation. During this phase parameters such as cells-cell ratio and culture media were tested and optimised in order to have functional hepatocytes and HSCs for three weeks in culture. During this time, the specific functional profile for each cell type, as well as viability, was accessed. At the end of the culture (day 21) hepatocyte-accessed functions in the 3D HepaRG/HSC co-culture were comparable or higher than the activities in 3D and 2D HepaRG mono-cultures. Conversely, fibrotic activation remained lower in co-cultures when compared with the 3D HSC mono-cultures. Besides being more representative of the *in vivo* situation, the 3D HepaRG/HSC co-cultures allow a better basal cellular state to induce compound-mediated HSC activation, especially when hepatocyte metabolisation is necessary.

In addition, techniques and strategies were developed to challenge the culture and to test whether the HSCs kept their capacity to activate, by mimicking liver injury. This injury was triggered by acetaminophen (APAP), which is metabolised via CYPs to the hepato-toxic compound NAPQI. As expected, the APAP dose-response curve reflects good hepatocyte-CYP activity but also an upregulation of the mRNA of ECM constituents, characteristic of fibrogenesis. This work was presented as a poster presentation at the **SEURAT-1** Annual Meeting in Barcelona.

At the moment, further characterisation of the model in terms of fibrotic outcomes and the testing of different compounds is being performed and will be submitted soon. In parallel, bioreactors have been developed that permitted successful drug-toxicity studies in 2D mono-cultures. After finalisation of phase II of the case study, maintenance of 3D co-cultures will be adapted to fit these devices and will be reported on in the next **SEURAT-1** Annual Report.

4.4.3 Selected Highlights: Bioreactor Developments and Characterisation of Hepatic Stellate Cells

Bioreactor Developments

The overarching goal of ***HeMiBio*** is to generate a microfluidic bioreactor that allows the maintenance of liver organoids for over 28 days *in vitro* so that chronic toxicity testing of cosmetics and pharmaceuticals can be undertaken. During year three of ***HeMiBio***, we have tested the second stage bioreactor (flow-over, see *Figure 4.12*), for its ability to maintain hepatic cells for prolonged durations, as well as an array of oxygen, glucose, and lactate sensors. The bioreactor designed and validated by the Nahmias and Jaeger teams permits the continuous monitoring of cell viability for over 28 days *in vitro* and a high-resolution analysis of hepatotoxicity. The reactor accurately predicted the TC50 values of acetaminophen, amiodarone, troglitazone, and rotenone, with an R^2 of 0.9 (data not shown). Bioreactors were sent to the Verfaillie, and van Grunsven teams in the consortium for further validation. A manuscript related to these findings as well as a patent application, has been submitted. A full description of the second stage bioreactor as well as the flow-through bioreactor will be included in the book describing progress made in year four of ***HeMiBio***.

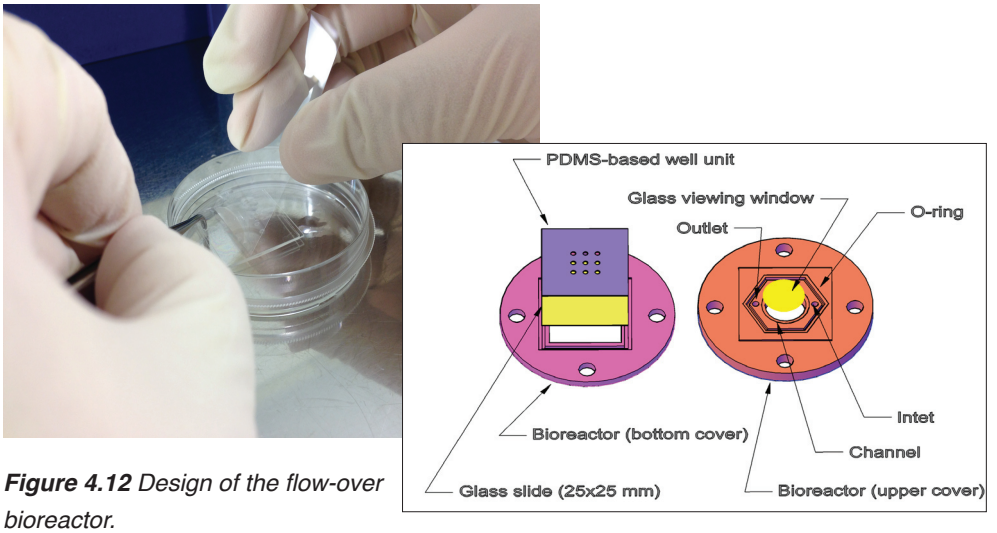

Figure 4.12 *Design of the flow-over bioreactor.*

Further Characterisation of Hepatic Stellate Cells

In order to maintain hepatic stellate cells (HSC) in an optimal phenotype in the bioreactor it is of utmost importance to understand the mechanisms controlling HSC activation. For that reason, quiescent (qHSC), activated HSC and liver sinusoidal endothelial cells (LSEC) were isolated from human non-parenchymal fractions and were assessed for their gene expression profile

and miRNA expression, together with DNA methylation profiling. The transcriptome analysis revealed that of the 20,216 genes examined, 0.46%, 0.44% and 5.1% were specifically expressed by qHSCs, LSECs and hepatocytes, respectively. Moreover, miRNA data analysis showed a massive over-expression in activated HSC compared to qHSC (80% upregulation), suggesting that miRNA (dys)-regulation could play an important role during HSC activation. In addition, by integrating miRNA-mRNA expression profiles we identified a set of deregulated miRNAs that present a significant correlation with the expression of their predicted target genes in activated HSC compared to quiescent HSC. Finally, more than 7% of the genes were found to be upregulated upon culture activation of HSCs, and the promoter methylome deviated dramatically from that of their quiescent counterparts.

In conclusion, our data provide the first gene and microRNA expression profiles as well as the first epigenetic pattern in human purified and uncultured liver cell types. Furthermore, the massive changes found in HSCs as a response to culture and activation appear to be, at least in part, reminiscent of that elicited in fibrotic liver.

4.4.4 Cross-Cluster Cooperation

HeMiBio organised a second joint meeting in Leuven, Belgium, on 10 September 2013. Investigators from *SCR&Tox*, DETECTIVE, NOTOX and COACH were invited to further discuss bioreactors as well as cell engineering for liver engineering purposes. This event stimulated a number of collaborations between ***HeMiBio*** partners and scientists from the other **SEURAT-1** projects, as outlined in a summary report given in section 4.11.9.

HeMiBio provided, and is still providing, input into several **SEURAT-1** Working Groups and activities, including the selection of cross-cluster standard 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 group of Vera Rogiers and Mathieu Vinken (Vrije Universiteit Brussel). Vinken 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 Brussel partner 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 many as fifteen standard operating procedures, describing methods related to functionality and drug-induced liver toxicity testing in cultured liver cells. These standard operating procedures will be consistently used by both consortia and, due to their inclusion in the ToxBank Data Warehouse, potentially other **SEURAT-1** projects. Also included in ToxBank are lists of drugs and cosmetics chemicals to be tested that have been compiled by the Vrije Universiteit Brussel group based on extended discussions with the DETECTIVE and

HeMiBio partners. In addition, the Vrije Universiteit Brussel 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 Brussel group is a member of the **SEURAT-1** Safety Assessment Working Group and co-leader of the **SEURAT-1** Mode-of-Action Working Group, both of which work towards the cluster-level objectives of the **SEURAT-1** Research Initiative. Moreover, in order to make data obtained within ***HeMiBio*** consortia available to **SEURAT-1** partners, raw data and analysed results have been uploaded to the ToxBank Data Warehouse. To date, ***HeMiBio*** partners from IDIBAPS, Vrije Universiteit Brussel, Medicyte GmbH and Universitetet i Oslo have uploaded data and procedures to the ToxBank Data Warehouse.

The following **SEURAT-1** workshops have been attended on behalf of ***HeMiBio***:

- **SEURAT-1** Stakeholder Event, 5 September 2013, in Brussels, Belgium (see summary report in section 4.12.3);
- **SEURAT-1** meets Tox21, 25–27 June 2013 in Ispra, Italy (see workshop report in section 5.3.1);
- Summer school of the DETECTIVE project. Mathieu Vinken: Introduction into drug-induced liver injury and adverse outcome pathways, 10–14 June in Slano, Croatia;
- Biokinetics workshop organised by Alexandre Pery, co-leader of the **SEURAT-1** Biokinetics Working Group, 24–25 September in Paris, France (see section 4.11.6).

4.4.5 Expected Progress within the Fourth Year

In year four of ***HeMiBio***, we will:

- Complete molecular engineering of PSC to allow cell tracing, conditional gene overexpression and for the inclusion of molecular sensors, and submit the data for publication;
- Complete optimisation of 3D cultures of hepatocytes, and hepatic stellate cells (from primary tissue, cell lines or pluripotent stem cells), and submit data for patent protection (if suitable) and publication;
- Complete functional and molecular/epigenetic characterisation of primary hepatic stellate cells from normal and cirrhotic livers, as well as following culture, and submit the data for publication;

- Complete methods to maintain or induce liver sinusoidal endothelial cell phenotype;
- Complete and submit toxicity data on **SEURAT-1** standard reference compounds testing of hepatocytes treated with the UpCyte® technology and PSC-derived hepatocytes in non-perfused conditions;
- Complete and submit results of the flow-over bioreactor;
- Integrate the sensor unit into the flow-over bioreactor;
- Validate the flow-through bioreactor.

4.4.6 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 should be transferrable to other bioreactors and should include: microfluidics and spatial isolation technologies; the development of sensor modules directed towards medium composition (pH, oxygen, glucose, etc.) as well as cell toxicity detection; and master stem cell lines allowing easy introduction of lineage-specific promoter constructs or toxicity detector gene sequences.

Current development of pancreatic bioreactor technology provide a good example. 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; however, 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. Pancreatic endoderm derived from hES cells has also efficiently generated glucose-responsive endocrine cells after implantation into mice (*Madsen, 2005; D'Amour et al., 2006; Zaret & Grompe 2008*). Thus, the selection of immature cells derived from hiPS cells and further differentiation in suitable 2D-/3D-bioreactors (that 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 is composed of approximately 1.2 million individual nephrons working in parallel. Each nephron can be divided into three main components: the glomerulus, the proximal tubule, and the loop of Henle. 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 to culture the three types of glomerular cells. For instance, the concept of a nephron-on-a-chip using a MEMS-based (Micro-Electro-Mechanical 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 the 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, their exposure to flow and certain oxygen levels, etc.). All these parameters can be integrated into a bioreactor system such as the one we propose here for the liver. The technologies developed in ***HeMiBio*** (i.e. cells that are manipulated so that their differentiation state, functionality and viability can be monitored, and 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 the use of animals.

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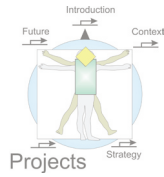
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4.5 DETECTIVE: Detection of Endpoints and Biomarkers for Repeated Dose Toxicity Using *in vitro* Systems

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4.5.1 Introduction and Objectives

As one of the building blocks of the **SEURAT-1** Research Initiative, the **DETECTIVE** project focuses on a key element on which *in vitro* toxicity testing relies: the development of robust and reliable, sensitive and specific *in vitro* biomarkers and surrogate endpoints that can be used for safety assessments of chronically acting toxicants relevant for humans.

Emphasis is on the systematic exploitation of a battery of complementary functional and '-omics' read-outs, including high-content and high-throughput screening platforms, to identify and investigate human biomarkers in cellular models for repeated dose *in vitro* testing. While functional parameters give more insights into the effects of toxicants on specific cell functions of interest, '-omics' techniques will deliver data on the entire cellular situation at the molecular level. Importantly, **DETECTIVE** performs, for the first time, an in-depth investigation of repeated dose effects on epigenetics and microRNA (miRNA) expressions, thus exploring whether such analyses deepen our understanding of toxic modes-of-action. In recent years, these two parameters have been identified as critical for cell behaviour and it will be a challenging task to determine whether the long-term application of chemicals affects cells at this level.

Biomarkers for predicting long-term toxicity in humans based on *in vitro* read-outs can be obtained by combining and subsequently integrating the various readouts. Relevant, specific, sensitive and predictive biomarkers will be selected based on integrative statistical analysis, systematic verification and correlation with *in vivo* data.

DETECTIVE concentrates on hepatotoxic, cardiotoxic and nephrotoxic effects representing three target organs of repeated dose toxicity. In addition, a biological model addressing repeated dose toxicity is being developed based on human embryonic stem cells (hESC). Ultimately, concepts developed should also be applicable to other organs or organ systems affected by systemic toxicants, such as the nervous system. Furthermore, it is expected that **DETECTIVE** will be able to define human toxicity pathways relevant for all organs.

The objectives in the third year of the **DETECTIVE** project were:

- To conduct functional and '-omics' experiments under optimised protocols with repeat dose exposures and recovery periods;
- To analyse data-rich '-omics' data;
- To define relevant biomarkers and adverse pathways with predictive values;
- To prepare a road map, including proof-of-concept case studies, in close collaboration with the other **SEURAT-1** projects.

In this report, we focus on the investigation of a new source for the development of biological models to be used for toxicity testing, which are human skin-derived precursor cells. This has been chosen as the highlight of the year in the **DETECTIVE** project and is reported in detail in section 4.5.3.

4.5.2 Main Achievements and Challenges in the Third Year

Coordination between **DETECTIVE** partners and other **SEURAT-1** projects has continued. As a result, three case studies were initiated in compliance with requests from COACH and in collaboration with other **SEURAT-1** projects (see also chapter 3). The case study proposals underwent a review process at the **SEURAT-1** level and by external reviewers and were ultimately approved.

In addition, exposure protocols were further optimised for long-term (up to two weeks) repeated dose exposures with recovery periods to investigate the reversibility of the effects of chosen toxicants. The protocols were validated for their applicability for proteomics, transcriptomics, epigenomics and metabonomics analysis. The optimal range of toxicant concentrations for '-omics' experiments was determined.

Furthermore, the applicability of human skin-derived precursor cells (hSKPs) and their hepatic differentiated progeny (hSKP-HPCs) as an *in vitro* model for hepatotoxicity testing was evaluated and confirmed. This was selected as the highlight from the third year (see section 4.5.3).

Regarding dissemination activities, the training plan was implemented and a summer school was organised in Slano, Croatia on 10-14 June, 2013. The **DETECTIVE** public website (www.detect-iv-e.eu) is regularly updated and the consortium achievements have been presented at scientific conferences, and also at the first **SEURAT-1** stakeholder event in Brussels on 5 September 2013 (see also section 4.12.3). Options for collaborations were evaluated and concluded during the joined **SEURAT-1** and Tox21 workshop in Ispra, 25–27 June 2013 (see section 5.3.1). The **DETECTIVE** partners have also submitted or published a range of peer-reviewed papers (see publication list at the end of this **DETECTIVE** report).

Functional Readouts

Standardised conditions were set up for both the MEA-based and xCELLigence impedance technology systems for measuring the effect of test substances on human iPS cell-derived cardiomyocytes. Impedance technology was also applied in the kidney model, assessing short- and long-term repeated dose toxic effects of selected compounds. Short- and long-term (up to 14 days of exposure) functional data were collected for different compounds with known cardiotoxicity effects. For automated high-resolution live cell imaging analysis, a range of BAC stress response reporter cell lines was generated. The reporter cell lines were analysed with various DILI compounds as well as with selected **SEURAT-1** standard reference compounds ('gold compounds') at diverse concentrations. A Methlab-based software pack was approved for automated quantification of hepatocyte polarity (bile canaliculi) by time lapse and fluorescent microscopy.

'-omics' Readouts

Gene expression profiling was conducted with hepatocytes, kidney cells and iPS cell-derived cardiomyocytes under repeated dose treatment settings; specific gene expression profiles were investigated by means of bioinformatics analysis of the gene array data. Signalling pathways triggered by ER-stress and JNK pathways in particular have been identified as crucial components of *in vivo* hepatotoxicity as well as *in vitro* and *in vivo* induced inflammation. Sets of specific gene clusters have been defined and can be used for rapid assessment of ER-stress or inflammation.

Whole genome DNA methylation, whole genome histone acetylation and miRNA data sets were generated for the kidney model. Within the liver model and the heart model, whole genome DNA methylation and miRNA data sets were generated and a range of biomarkers in these target organ models were identified.

Additionally, novel metabonomic protocols for the **DETECTIVE** cell test systems were

established and validated for both NMR and GC-MS. The established protocols were used for metabonomic profiling of toxic stress responses in cardiomyocytes and renal proximal tubule epithelial cells. A large amount of metabolite-profiling data has been generated for repeated dose toxicity studies of the two cell systems and a range of toxicity-related metabolites was identified in both media and intracellular metabolite extract samples.

Integration of Biomarkers

The raw database has been established and is ready for use by project partners and members of other **SEURAT-1** projects. The database is continuously updated with the experimental data from **DETECTIVE**. Collected data are curated locally and then uploaded into the ToxBank Data Warehouse. The database development was coordinated with ToxBank. To make the connection with ToxBank possible, the data structures are based on the ISATAB format. Preliminary integrative analysis of epigenetics and transcriptomics data was performed.

4.5.3 Selected Highlight: Human Skin-derived Precursors as a Novel Cell Source for Evaluating the Hepatotoxic Potential of Chemicals

State-of-the-Art

Since the liver is a first-line target organ during toxic assault, human-based hepatic cell systems are valuable *in vitro* tools to study the potential hepatotoxicity of chemical substances. Freshly isolated human hepatocytes represent the model that most appropriately reflects the *in vivo* situation and are considered to be the gold standard in liver-based *in vitro* modelling (*Guguen-Guilluozo & Guillouzo, 2010*). However, due to intensive transplantation programs, hepatocytes can be seldom isolated from healthy human livers. Instead, they are obtained from patients suffering from severe liver injuries or coping with a multi-drug regimen; this often results in poor cell quality (*Guguen-Guilluozo & Guillouzo, 2010*). Immortalised human liver cell lines, including HepG2 (*Schoonen et al., 2005; Jennen et al., 2010*), Fa2N-4 (*Mills et al., 2004*) and HepaRG (*Guillouzo et al., 2007*) have become increasingly popular as *in vitro* models to study human liver function and toxicity. These cell lines are readily available and can be kept in culture for long periods of time, but they suffer from genotypical instability and decreased (or even absent) metabolic activity. In addition, currently available cell lines do not represent population diversity (*LeCluyse et al., 2012*).

New developments in stem cell research might create new possibilities as stem cells represent a virtually inexhaustible cell source and have the ability to differentiate in multiple cell types. The establishment of continuous cell lines from embryonic stem cells (ESC) (*Thomson et*

al., 1998) and induced pluripotent stem cells (iPSC) (*Takahashi & Yamanaka, 2006*), the advancements in isolating and culturing adult stem cells (ASC) (*Jiang et al., 2002*) and in particular the breakthrough in differentiating stem cells into cells with particular functionalities brought huge expectations to the scientific and industrial community.

In this study, human skin-derived precursors (hSKP), isolated from human (fore)skin, and their hepatic derivatives are investigated (*Toma et al., 2001; Biernaskie et al., 2006; Jinno et al., 2010*). These cells have a high self-renewal and high multipotent differentiation capacity. hSKP can be directed towards the hepatic lineage (*De Kock et al., 2009; 2011; 2012*) upon sequential exposure to growth factors and cytokines that mimic liver development *in vivo* (*Snykers et al., 2006*). The obtained hepatic-differentiated hSKP could represent a novel *in vitro* model for hepatotoxicity screening of chemical substances. Acetaminophen (acetyl-para-aminophenol, APAP), which is a common over-the-counter analgesic considered to be safe when used at therapeutic doses, is used here as a proof-of-principle reference compound. When taken in overdose, APAP becomes hepatotoxic and can cause acute liver failure (ALF), the latter being a leading cause of drug-induced liver injury (DILI) (*James et al., 2003*). The toxic mechanism of APAP in adult hepatocytes is known to involve several toxicity pathways (McGill et al., 2012; Zimmermann & Maddrey, 1995). It is acknowledged that APAP is metabolically activated to N-acetyl-p-benzoquinone imine (NAPQI), which is normally detoxified by glutathione. Depletion of glutathione following APAP overdose leads to NAPQI accumulation that adversely binds to different cellular proteins, causing toxicity. Here, we evaluate to what extent APAP exposure modulates the whole genome expression of hepatic-differentiated hSKP versus primary human hepatocytes by using a full genome microarray platform.

Approach

Isolation, cultivation and hepatogenic differentiation of hSKP: hSKP were isolated from foreskin circumcision samples of one- to ten-year-old boys after the informed consent of the parents. The cells were isolated as previously described in *De Kock et al.* (*2012*). Subsequently, hSKP were seeded on collagen type 1-coated 24-well plates and T75 culture flasks and cultured until 90% confluence in Basal Medium (BM) was reached. BM consisted of DMEM+GLUTAMAX/F12 Nutrient Mixture supplemented with 7.33 IU/mL benzyl penicillin, 50 mg/mL streptomycin sulfate, 2.5 mg/mL fungizone, 0.1 mM L-ascorbic acid, 4 mg/L L-nicotinamide, 1 mg/mL linoleic acid-albumin and 27.3 mg/mL sodium pyruvate. The differentiation protocol, which consisted of sequential exposure of hSKP to hepatogenic growth factors and cytokines, was started when the cells reached 90% confluence (*Figure 4.13*). The differentiation was completed after 24 days. Then the 24-hour IC10 (10% inhibitory concentration) value for APAP was determined by a cell viability assay.

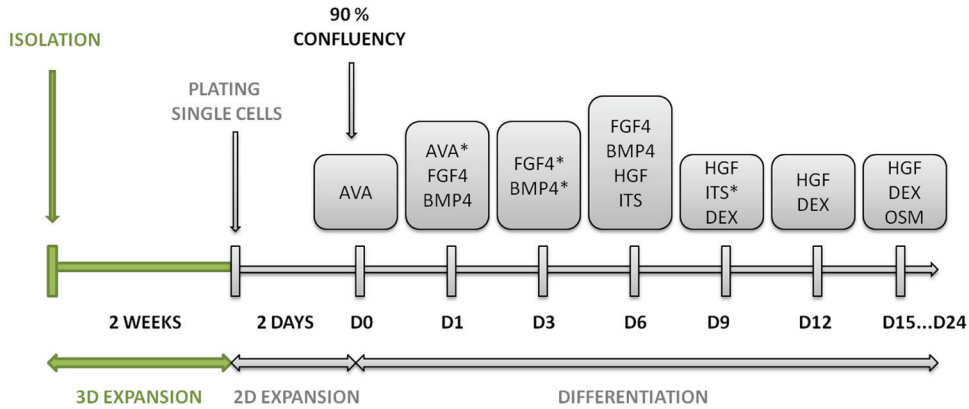

Figure 4.13 *Hepatogenic differentiation of hSKP (AVA: 50 ng/mL activin A; AVA*: 25 ng/mL activin A; FGF4: 5 ng/mL fibroblast growth factor 4; BMP4: 10 ng/mL bone morphogenetic protein 4; FGF4*: 10 ng/mL fibroblast growth factor 4; BMP4*: 20 ng/mL bone morphogenetic protein 4; HGF: 30 ng/mL hepatocyte growth factor; ITS: 0.5% (v/v) insulin-transferrin-sodium selenite; ITS*: 0.25% (v/v) insulin-transferrin-sodium selenite; DEX: 0.02 µg/mL dexamethasone; OSM: 10 ng/mL oncostatin M).*

Microarray data analysis: Human Genome U133 plus 2.0 arrays from Affymetrix were used for whole genome expression analysis. Microarray data of human hepatocyte cultures (hHEP) established from human cryopreserved hepatocytes were obtained from the toxicology database TG-GATEs (*Urushidani, 2005; Uehara et al., 2010*).

Results

Characterisation of hSKP-derived hepatic progeny: After 24 days of sequential exposure to hepatogenic growth factors and cytokines, hSKP undergo a transition towards the hepatic lineage. As illustrated by the PCA plots shown in *Figure 4.14A*, hepatic differentiated hSKP, further referred to as hSKP-derived hepatic progenitor cells (hSKP-HPC), shift towards human hepatocyte cultures (hHEP) and human liver samples (LIVER).

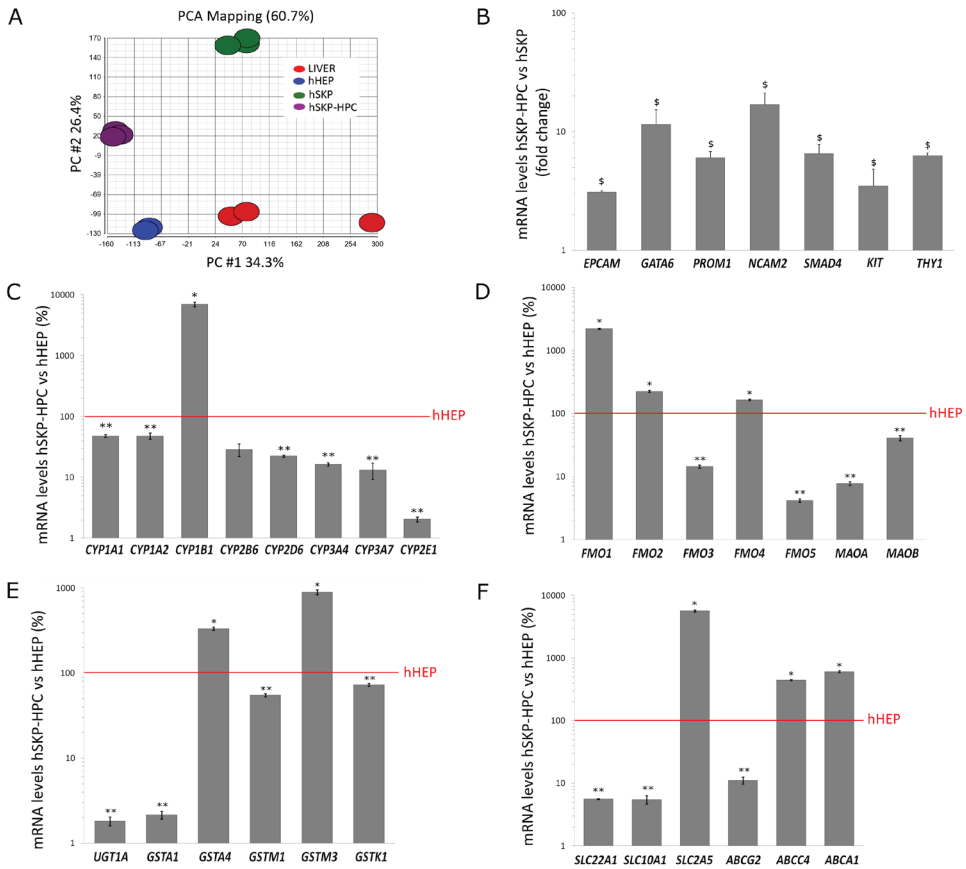

Figure 4.14 *Microarray analysis: hSKP-HPC express hepatic progenitor cell markers as well as specific markers of mature hepatocytes. hHEP is arbitrarily set to 100%. $ Significantly increased gene expression in hSKP-HPC vs. undifferentiated hSKP (fold change > 2; TTEST p-value<0.05). * Significantly increased gene expression in hSKP-HPC vs hHEP (fold change > 2; Student's t-test p-value<0.05). ** Significantly decreased gene expression vs hHep (fold change < 2; Student's t-test p-value < 0.05).*

Further analysis of hSKP-HPC shows that, compared to undifferentiated hSKP, these cells express significantly higher levels of typical hepatic progenitor cell markers including epithelial cell adhesion molecule (EPCAM), GATA motif binding protein 6 (GATA6), prominin 1 (PROM1), neural cell adhesion molecule (NCAM), SMAD family member 4 (SMAD4), stem cell factor receptor (KIT) and thymocyte differentiation antigen 1 (THY1) (*Figure 4.14B*). Comparison between hSKP-HPC and hHEP, with respect to the expression levels of typical hepatic markers, shows that the major cytochrome P450 enzymes (CYP) are expressed at

a lower level in hSKP-HPC than in hHEP (*Figure 4.14C*). CYP1A1 and CYP1A2 reach 48% of the hHEP expression levels and CYP2D6, CYP3A4, CYP3A7 and CYP2E1 get to 23%, 16%, 13% and 2%, respectively. Interestingly, the expression of CYP1B1 is 100-fold higher in hSKP-HPC than in hHEP (*Figure 4.14C*). The expression of the FMO1, which is a typical fetal liver phase I enzyme, is ten times higher in hSKP-HPC than in hHEP (*Figure 4.14D*). FMO2 and FMO4, which are generally found in adult human livers, achieve levels comparable to those of hHEP (*Figure 4.14D*). In contrast, FMO3 expression stays 10 times lower than in hHEP (*Figure 4.14D*). The gene expression of monoamine oxidases A and B (MAOA, MAOB), another class of phase I oxidation enzymes, stays lower in hSKP-HPC than in hHEP (*Figure 4.14D*). The phase II hepatic enzymes glutathione S-transferase GSTA4 and GSTM3 are found to be highly expressed in hSKP-HPC and reach levels that are respectively three- and nine-fold higher than those found in hHEP (*Figure 4.14E*). Other phase II enzymes (i.e. GSTM1 and GSTK1) reach 52% and 72% of the expression levels of hHEP, respectively. UGT1A and GSTA1 expression is dramatically lower in hSKP-HPC than in hHEP (*Figure 4.14E*). Typical phase 0 hepatic uptake drug transporters, including the solute carrier families 10A1 (SLC10A1 or NTCP) and SLC22A1 (OCT1), are barely expressed at the gene level in hSKP-HPC. Instead, the expression of other influx transporters, such as SLC2A5, is up to 50 times higher in hSKP-HPC than in hHEP (*Figure 4.14F*). Phase III efflux transporters, including ATP-binding cassette G2 (ABCG2 also referred to as BCRP), ABCC4 (MRP4) and ABCA1, known to be present in both adult and perinatal liver, are also expressed at significantly higher levels in hSKP-HPC (*Figure 4.14F*).

Predictive capacity of hSKP-HPC for APAP-induced hepatotoxicity: Sub-cytotoxic concentrations of APAP were determined by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) viability test after exposing hSKP-HPC to a range of APAP concentrations for 24 hours. An average IC10 value of 18 mM was calculated from a total of three tests. Principle Component Analysis (PCA) of the microarray data shows highly reproducible results for the *in vitro* samples (*Figure 4.15*). hSKP-HPC and hHEP exposed to IC10 of APAP (hSKP-HPC+APAP and hHEP+APAP), as well as the respective control samples, cluster individually. From the same PCA plot it can also be observed that APAP exposure results in a more pronounced shift of hSKP-HPC as compared to hHEP. Upon APAP exposure, a total of 5591 genes is significantly (Student t-test with p-value <0.05) modulated (minimal 2-fold) in hSKP-HPC, out of which 2646 and 2945 genes are up- or downregulated, respectively. In hHEP, only 511 and 868 genes are significantly up- and downregulated, respectively (minimal 2-fold; Student t-test with p-value <0.05).

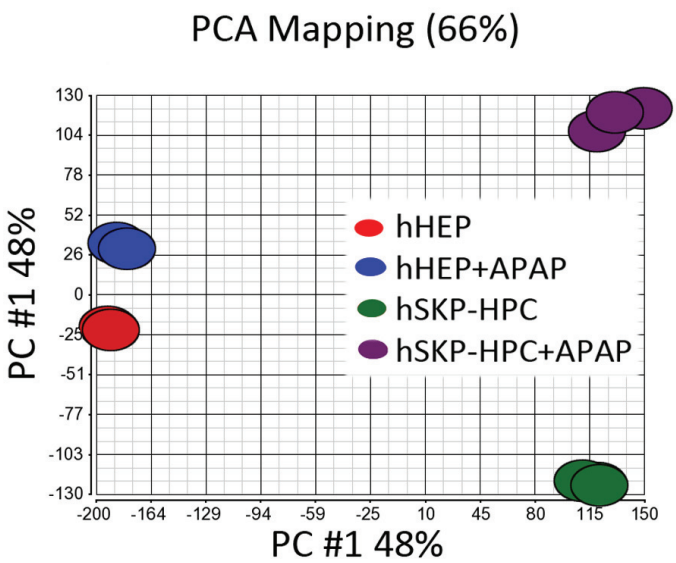

Figure 4.15 *PCA-plots of the microarray data of hSKP-HPC and hHEP exposed to APAP versus control.*

Functional analysis of the differentially expressed genes reveals the enrichment of gene classes of specific toxicological functions. As such, five liver-related "Toxicological Gene Classes" (Liver Damage, Liver Proliferation, Liver Necrosis/Cell Death, Liver Steatosis and Liver Hepatitis) could be identified with high accuracy (Fisher's Exact p-value < 0.05) in both hSKP-HPC and hHEP exposed to APAP (*Table 4.7*).

Table 4.7 *Enrichment of Toxicological Gene Classes of in vitro samples following exposure to APAP.*

TOXICOLOGICAL CLASSES	PERCENTAGE OF MODULATED GENES	P-VALUE (FISHER'S EXACT)	PERCENTAGE OF MODULATED GENES	P-VALUE (FISHER'S EXACT)
	HSKP-HPC + APAP	HHEP + APAP	HSKP-HPC + APAP	HHEP + APAP
LIVER DAMAGE	11% (37/349)	1.78E-03	5% (18/349)	4.96E-03
LIVER PROLIFERATION	19% (53/286)	9.48E-03	9% (26/286)	4.08E-04
LIVER NECROSIS / CELL DEATH	11% (55/483)	1.71E-02	5% (25/483)	3.95E-03
LIVER STEATOSIS	16% (55/337)	3.00E-02	7% (23/337)	4.06E-02
LIVER HEPATITIS	7% (25/343)	3.57E-03	5% (16/343)	7.29E-03
LIVER STEATOHEPATITIS	17% (11/66)	3.00E-02	2% (1/66)	2.30E-01
LIVER EDEMA	40% (2/5)	4.13E-02	-	-

The hSKP-HPC+APAP data could also identify two relevant toxicological functions (Liver Steatohepatitis and Liver Edema). The percentage of modulated genes in each function was consistently higher in the hSKP-HPC+APAP samples than in the hHEP+APAP samples.

The identified toxicological classes can be further divided into sub-functions, although these sub-functions do not always correlate among hSKP-HPC+APAP and hHEP+APAP (*Table 4.8)*. More specifically, the Liver Damage gene class is divided into four sub-functions in hSKP-HPC+APAP and two sub-functions in hHEP+APAP. The Liver Proliferation Class, on the other hand, has two common sub-functions in both cell types, but an extra three sub-functions in hHEP+APAP. Six sub-functions of the Liver Necrosis/Cell Death Class are enriched in hSKP-HPC+APAP and four in hHEP+APAP. Only one of these, however, is common to both cell systems. Four sub-functions of Liver Hepatitis are identified in hHEP+APAP, out of which one is common to hHEP+APAP and hSKP-HPC+APAP. Liver Steatosis is composed of two sub-functions, of which each is identified by a different cell system. The number of modulated genes of each sub-function is consistently higher in hSKP-HPC+APAP compared to hHEP+APAP, resulting in a higher sub-function enrichment in the former cell type.

Table 4.8 *Enriched sub functions of Toxicological Gene Classes in in vitro samples following exposure to APAP.*

	SUB FUNCTIONS	HSKP-HPC + APAP	P-VALUE (FISHER'S EXACT)	HHEP + APAP	P-VALUE (FISHER'S EXACT)
LIVER DAMAGE	DAMAGE OF LIVER CELLS	23.3% (10/43)	1.78E-03		
	DAMAGE OF HEPATOCYTES	25.0% (7/28)	2.16E-03		
	INJURY OF LIVER CELLS	19.0% (4/21)	7.14E-03		
	INJURY OF HEPATOCYTES	20.0% (3/15)	2.85E-02		
	DAMAGE OF LIVER			5.9% (18/304)	4.96E-03
	INJURY OF LIVER			5.6% (11/195)	3.49E-02
LIVER PROLIFERATION	PROLIFERATION OF HEPATOCYTES	19.4% (39/201)	9.48E-03	9.1% (18/201)	1.94E-03
	PROLIFERATION OF LIVER CELLS	17.8% (47/264)	1.17E-02	8.7% (23/264)	4.08E-04
	PROLIFERATION OF HEPATIC STELLATE CELLS			8.6% (6/70)	3.07E-02
	QUANTITY OF HEPATOCYTES			14.3% (5/35)	8.72E-03
	ARREST IN GROWTH OF HEPATOCYTES			66.7% (2/3)	1.15E-02
LIVER NECROSIS / CELL DEATH	APOPTOSIS OF LIVER CELLS	14.0% (40/285)	1.71E-02		
	APOPTOSIS OF HEPATOCYTES	13.4% (33/247)	2.24E-02		
	CELL DEATH OF LIVER CELLS	12.4% (49/395)	2.28E-02	5.3% (21/395)	6.26E-03
	CELL DEATH OF HEPATOCYTES	12.0% (38/316)	2.80E-02		
	APOPTOSIS OF SINUSOIDAL ENDOTHELIAL CELLS	100.0% (2/2)	4.13E-02		
	DELAY IN CELL DEATH OF HEPATOCYTES	50.0% (2/4)	4.13E-02		
	NECROSIS OF LIVER			5.3% (25/473)	4.83E-03
	APOPTOSIS OF LIVER CELL LINES			14.3% (6/42)	3.95E-03
	APOPTOSIS OF HEPATIC STELLATE CELLS			18.2% (4/22)	1.24E-02
LIVER HEPATITIS	ALCOHOLIC HEPATITIS	38.2% (13/34)	3.57E-03	14.7% (5/34)	3.83E-02
	NONALCOHOLIC STEATOHEPATITIS	20.0% (9/45)	3.00E-02		
	CHRONIC HEPATITIS C			23.5% (16/68)	2.23E-02
	CHOLESTATIC HEPATITIS			9.7% (3/31)	7.29E-03
	CHRONIC AUTOIMMUNE HEPATITIS			33.3% (2/6)	2.20E-02
LIVER STEATOSIS	NONALCOHOLIC STEATOHEPATITIS	20.0% (9/45)	3.00E-02		
	HEPATIC STEATOSIS			6.5% (20/309)	4.06E-02

When looking at the gene level, it is found that a number of genes of the previously identified 'Toxicological Classes' are commonly modulated in both hSKP-HPC and hHEP exposed to APAP. Five genes, including Bcl-2 interacting mediator of cell death (BCL2L11), cellular oncogene c-fos (FOS), heme oxygenase 1 (HMOX1), TIMP metallopeptidase inhibitor (TIMP) 3 and aryl hydrocarbon receptor (AHR), are significantly upregulated (fold change >2, Fisher's Exact p-value < 0.05) and three genes, including insulin-like growth factor (IGF) 1, regucalcin (RGN) and inhibin beta A (INHBA), are significantly downregulated (fold change >2, Fisher's Exact p-value < 0.05) (*Figure 4.16*). These results were confirmed by qPCR analysis for both hSKP-HPC and hSKP-HPC + APAP samples, validating the obtained microarray data.

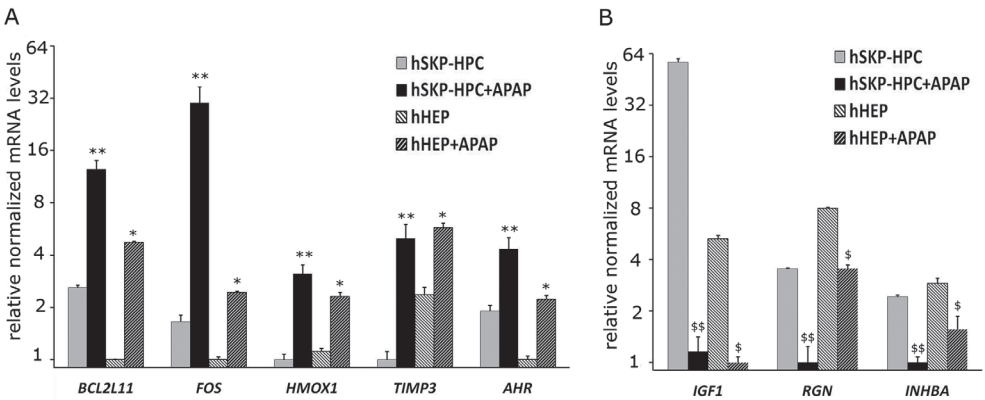

Figure 4.16 *Microarray expression of Toxicological Classes genes that are commonly modulated in hSKP-HPC and hHEP exposed to APAP. (A) Common upregulated genes. (B) Common downregulated genes. ** Significantly increased gene expression of hSKP-HPC exposed to APAP vs unexposed hSKP-HPC (fold change > 2; Fisher's Exact p-value < 0.05). * significantly increased gene expression of hHEP exposed to APAP vs unexposed hHep (fold change > 2; Fisher's Exact p-value < 0.05). $$ Significantly decreased gene expression of hSKP-HPC exposed to APAP vs unexposed hSKP-HPC (fold change > 2; Fisher's Exact p-value < 0.05). $ Significantly decreased gene expression of hHEP exposed to APAP vs unexposed hHep (fold change > 2; Fisher's Exact p-value < 0.05).*

Conclusion

Upon exposure to hepatogenic growth factors, hSKP acquire specific features of hepatic progenitor cells, as well as typical characteristics of adult hepatocytes, such as key biotransformation enzymes and drug transporters. Differentiated hSKP (hSKP-HPC) respond to acetaminophen exposure in a comparable way as primary human hepatocytes in culture, as illustrated by toxicogenomics analysis. The toxicological responses 'liver damage', 'liver

proliferation', 'liver necrosis' and 'liver steatosis' are found to be significantly enriched in both *in vitro* models. Genes associated with either cytotoxic responses or induction of apoptosis (BCL2L11, FOS, HMOX1, TIMP3 and AHR) are commonly upregulated and might represent potential molecular biomarkers for hepatic toxicity. In conclusion, our data provides a first indication that human skin stem cell-derived hepatic cells could be valuable tools in the early *in vitro* prediction of hepatotoxicity and could therefore alleviate the necessity for scarce human primary hepatocyte cultures.

4.5.4 Innovation

The main innovation of the selected research highlighted in this report includes hepatic progenitor cells derived from hSKP. The hepatic progenitor cells acquire features of adult hepatocytes and express key biotransformation enzymes and drug transporters. The hepatic progenitor cells manifest toxicological responses similar to adult hepatocytes, e.g. regarding damage, proliferation, necrosis and steatosis associated genes. A range of biomarkers representing stress responses was verified and is induced in both primary human hepatocytes and in the hepatic progenitor cells. The hSKP cells represent the first example of a cell system based on a ethically non-controversial adult stem cell source, which is suitable for the assessment of liver toxicity. The cells can be expanded significantly and could provide almost unlimited cell supply suitable for high-throughput toxicity profiling. The cells can be obtained from different donors with desired genotype and therefore they could be potentially applicable for addressing genetic diversity relevant issues.

4.5.5 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.

When 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 sections 4.11.2-4.11.8), **DETECTIVE** has shown active participation in four of the six working groups. Partners of the **DETECTIVE** consortium are co-leaders in the Data Analysis Working Group, Gold Compounds Working Group, Stem Cells Working Group and Mode-of-Action Working Group. **DETECTIVE** 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.

One part of the **DETECTIVE** project 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 **DETECTIVE** partners are converted to ISATAB format and sent to the ToxBank Data Warehouse.

On-going 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.

In cooperation with *SCR&Tox*, **DETECTIVE** has optimised differentiation protocols for cardiomyocyte generation, one of **DETECTIVE's** founding cell models.

DETECTIVE expertise in generating pathway-specific reporter constructs was shared with *HeMiBio* and *SCR&Tox* in order to accelerate generation of new sensor cell lines for toxicity screening. **DETECTIVE** is engaged in strong cross-consortia cooperation for planning and execution of **SEURAT-1** case studies and is collaborating with both COSMOS and ToxBank in a 'read-across' case study, as well as with *SCR&Tox* in the case study *Prediction of delayed organ specific toxicity.*

4.5.6 Expected Progress within the Fourth Year

The focus of **DETECTIVE** in the following year will be on integrated data analysis of data-rich '-omics' and high-throughput imaging experiments. Additional sets of carefully chosen compounds will be used for this purpose. The analysis will be supported by expertise, software tools and additional data resources from other **SEURAT-1** projects. The main aim is the determination of organ-specific and general biomarkers of adverse pathways with chronic toxicity predictive values. Experiments for three proof-of-concept case studies will be conducted:

- Read-across case study, particularly focusing on the use of biomarkers from '-omics' investigations to support read-across;
- Prediction of delayed cardiotoxicity, neurotoxicity and hepatoxicity, via cell type-specific and -unspecific mechanisms;
- Challenging the predictive power and robustness of an adverse outcome pathway construct from bile salt export pump inhibition to cholestatic injury.

4.5.7 Future Perspectives

Successful completion of the **DETECTIVE** project will change our understanding of repeated dose toxicity testing methods. This will lead 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. This will lay the foundation for subsequent efforts in follow-up activities at the completion of the **SEURAT-1** Research Initiative. Such future activities 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 toxic 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 the detection of endpoints and biomarkers for repeated dose toxicity, derived by the end of the **DETECTIVE** project, will help to establish a detailed proof-of-concept-based 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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4.6 COSMOS: Integrated *in Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety

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4.6.1 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 4.17*.

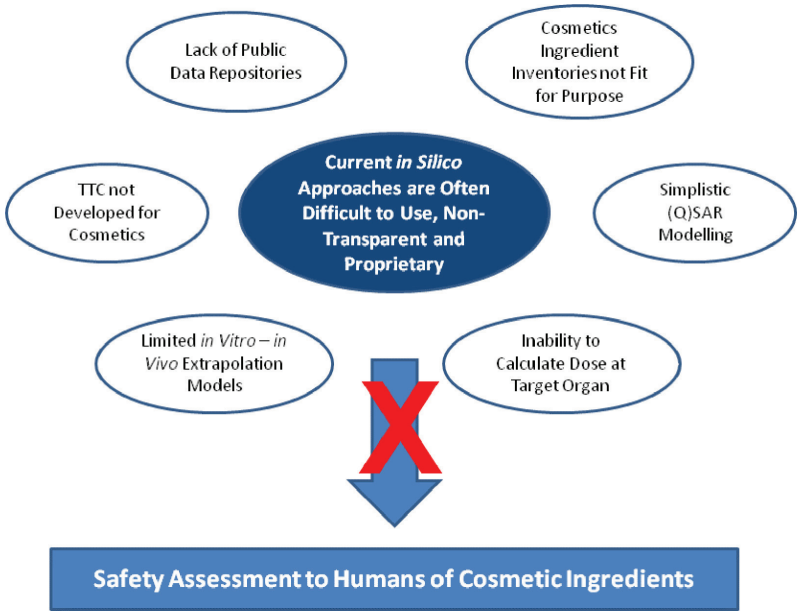

Figure 4.17 *Summary of the knowledge gaps preventing the assessment of the safety of cosmetic ingredients to humans from computational techniques.*

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 and enable extrapolation 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 (Adverse Outcome Pathways – AOPs). 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;

➠ Extend the TTC approach and assess its applicability to cosmetics;

➠ Develop innovative toxicity prediction strategies based on chemical categories, read-across and QSARs for organ level toxicity and relate these to key events in adverse outcome pathways (AOPs);

➠ Develop a multi-scale modelling approach including cell-based and physiologically-based 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 the COSMOS Database and Threshold of Toxicological Concern approach for cosmetics-related substances will be highlighted as major achievements of the third project year (see sections 4.6.3 and 4.6.4, respectively).

Overall, **COSMOS** is contributing to the objectives of the **SEURAT-1** Research Initiative by:

➠ Building a toxicity database and compiling a cosmetic materials inventory;

➠ Updating the TTC approach for cosmetic ingredients;

➠ Developing AOP-derived models for organ-level toxicity;

➠ Creating biokinetics models to assist in *in vitro* to *in vivo* extrapolation (IVIVE).

Key Deliverable 1: Toxicity database and COSMOS Cosmetics Inventory. The **COSMOS** database of toxicological information for cosmetic ingredients (and beyond), including the COSMOS Cosmetics Inventory, provides the backbone to 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 has the capability of contributing to the development of alternatives in the other **SEURAT-1** projects by access to high quality data as well as to the **SEURAT-1** case studies, such as the cross-cluster case study on read-across (see section 3.5.1). COSMOS DB version 1.0 was released publicly in December 2013.

Key Deliverable 2: 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 safety assessment. The TTC approaches have updated current knowledge and will be supported by the capability to build them on mechanistic knowledge.

Key Deliverable 3: 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 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 mode-of-action knowledge.

Key Deliverable 4: Biokinetics models to assist in IVIVE. Models for toxicodynamics and toxicokinetics are being developed within **COSMOS** which extend capabilities for *in vitro* – *in vivo* extrapolation (IVIVE), allowing for the better application of results from cell based assays to perform human safety assessment. Research includes (i) kinetics modelling (e.g. through physiologically-based pharmacokinetic (PBPK) models); (ii) a better understanding of the effect of the test system (e.g. sorption) and chemicals (e.g. volatility, stability) properties relating to extrapolation; and (iii) 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.

4.6.2 Main Achievements and Challenges in the Third Year

The third year of the project has resulted in a number of significant results from **COSMOS**. The main findings are summarised in the following.

COSMOS Inventory and Database

The COSMOS Cosmetics Inventory is a compilation of cosmetics-related ingredients incorporating information from the European Commission's Cosmetic Ingredients (CosIng) database and the US Personal Care Products Council (PCPC) lists, including over 19,000 substances. Linked to the inventory, the COSMOS Database (DB) is a comprehensive, reliable, relational database. COSMOS DB is completely based on open source technology.

COSMOS DB version 1.0 was made publicly available in December 2013 from the URL http://cosmosdb.cosmostox.eu. A webinar explained the use and application of the Database, the recording and a short user guidance document are available from the **COSMOS** website

(http://www.cosmostox.eu/what/COSMOSdb). COSMOS DB links chemical structures to repeated dose toxicity, skin permeability and other endpoint data. In total, COSMOS DB v1.0 contains more than 12,000 toxicity studies across 27 endpoints for over 1,600 compounds. More than 80,000 chemical records with more than 44,000 unique structures are flexibly searchable by name, CAS number, graphical representation, SMILES strings or other identifiers.

COSMOS DB also contains a Data Entry System for the inclusion of further chemical structures and toxicity data. The high quality of chemical records/structures and toxicity data within COSMOS DB was assured via formal quality control (QC) and quality assurance (QA) procedures. Toxicity data were assessed for their quality and this information is also available in COSMOS DB. The data record reliability was rated objectively by applying **COSMOS** MINImum Study (MINIS) criteria, which represent the set of required and recommended experimental parameters. Data acceptance was assessed by toxicologists using Klimisch scores.

In order to ensure wide uptake of COSMOS DB, it is being widely disseminated including through other international projects. COSMOS DB is supported by COSMOS Space which facilitates sharing of predictive toxicology resources (data sets, models, workflows, documentation, meta-data as wikis editable by the data owners). COSMOS DB forms a robust platform to mine data and thus supports risk assessment within the 21st Century Toxicology and AOP frameworks.

Threshold of Toxicological Concern (TTC) Approach for Cosmetic Substances

The **COSMOS** project has supported the evaluation and extension of the Threshold of Toxicological Concern (TTC) approach for the safety assessment of cosmetics-related chemicals. Fundamental to this has been the compilation of a new oral repeated-dose toxicity database, oRepeatTox DB, as a resource to construct the new **COSMOS** non-cancer TTC database of No Observable (Adverse) Effect Levels (NO(A)ELs). The quality of the relevant TTC database was reviewed by a group of independent experts (external to the **COSMOS** project) co-ordinated as an ILSI-EU Expert Group. The quality of the studies used in the review sessions was assigned with Klimisch scores. The **COSMOS** curation strategy and process as well as the outcomes from these expert reviews will be documented within the final COSMOS TTC database. The database has been mapped onto cosmetics-related chemical space and use categories as defined in the COSMOS Cosmetics Inventory, and analysed according to the Cramer Classes and for various compound classes. Based on the new COSMOS TTC database and the ILSI-EU Expert Group's recommendations on how to use the database, a new non-cancer TTC dataset is being finalised and be made publicly available.

Oral-to-dermal extrapolation issues have been addressed in collaboration with a second ILSI-EU Expert Group. To support the evaluation, a new **COSMOS** skin permeability database

has been developed and will be implemented in COSMOS DB. These data along with bioavailability models have supported the design of a tiered decision tree workflow to address exposure scenarios of chemicals in cosmetics products and bioavailability issues relevant to cosmetics.

Computational Tools for Toxicity Prediction

A number of computational (so-called *in silico)* models have been developed and evaluated within the **COSMOS** project. The state-of-the-art of the models for the prediction of chronic toxicity was reviewed, with the outcomes of the analysis directing model development in **COSMOS**. Innovative models have been developed for various endpoints including long-term and target organ toxicity, dermal and oral absorption and kinetic and metabolic (skin/liver) studies. Moreover, grouping approaches for toxicity prediction have been developed and applied with an emphasis on hair dyes. Data mining of COSMOS oRepeatTox DB has identified structural fragments capable of inducing hepatotoxicity; this knowledge has been captured in the form of 'chemotypes' relevant for liver toxicity (steatosis, steatohepatitis, fibrosis) of cosmetics-related chemicals. The studies were supported by molecular modelling methodologies applied to predict the binding to two nuclear receptors considered to be involved in liver steatosis (LXR and PPARγ). In order to support the oral-to-dermal extrapolation, for example relevant to TTC, quantitative structure-activity relationship (QSAR) models for dermal absorption have been developed.

Toxicokinetics

Open source software tools were also developed in the **COSMOS** project to simulate the long-term (repeat exposure) toxic effects of chemicals, including substances in cosmetics and personal care products, in *in vitro* systems. The approach is based on the previously developed Virtual Cell Based Assay (VCBA), re-coded and re-implemented in the open-source KNIME platform. It is designed for modelling *in vitro* experiments by taking into account the chemical fate within the *in vitro* test system, the cell dynamics and the toxicological effects of the chemical on the cell population. Another tool developed was a model for human bioconcentration factor.

In order to support *in vitro* to *in vivo* and route-to-route (oral to dermal and inhalation to dermal) extrapolations, many physiologically-based toxicokinetic (PBTK) models were calibrated for cosmetic ingredients and drugs. The PBTK models were refined to take account of uptake in different tissues (gastrointestinal tract, skin, lungs) and a methodology to calibrate these models without animal testing was proposed. PBTK models combined with cell based assays (incorporating aspects of chemical fate, cell growth, toxicity and feedback) enable realistic estimates of *in vivo* concentration (organ level) from *in vitro* data.

This methodology was supplemented with QSAR models to predict hepatic clearance. In addition, a 2D liver model was developed which included mechanisms for cell necrosis and cell proliferation. This allowed for the analysis of the effect of the accumulation of compounds on hepatocyte viability and detoxification capacity after long-term repeated exposure.

COSMOS KNIME Software and Workflows

Computational models developed in the **COSMOS** project have been coded using the open access KNIME workflow technology. The models implemented in KNIME include (i) mechanistic profilers, e.g. for protein binding potential and liver toxicity; (ii) the Virtual Cell Based model; (iii) a KNIME workflow for *in vitro* to *in vivo* extrapolation; (iv) a skin permeability (K_P) prediction model adapted from *Potts & Guy (1992)*; (v) metabolism simulation of chemicals in liver and skin.

In order to support the presentation of the models, the KNIME desktop applications have been extended in a number of significant ways. The capability to archive various (development) versions of workflows has been introduced in the KNIME server in order to reproduce results created with previous workflow versions. Recent improvements, along with those in the KNIME WebPortal, have allowed not only for the implementation of more models developed within **COSMOS** into KNIME but also better end-user experience and performance. Workflows can be executed from locally installed KNIME software or via a web browser using the KNIME WebPortal. The latter is accessible through the **COSMOS**-linked URL http://knimewebportal.cosmostox.eu.

Contributions to SEURAT-1 Case studies

COSMOS work on computational predictive models feeds into the case studies as well as the COSMOS Database.

COSMOS is leading the case study on developing chemotypes for mitochondrial toxicity (see section 3.4.5). The aim of this case study is to develop an *in silico* profiler consisting of a series of chemotypes to identify compounds with the ability to induce mitochondrial toxicity. To identify chemotypes associated with toxicity to mitochondria within the datasets investigated, the ToxPrint library of molecular fragments was used within the freely available ChemoTyper software (https://chemotyper.org). A fragment was deemed to be related to mitochondrial toxicity if it identified two or more chemicals, of which at least 80% of the chemicals identified by the fragment were associated with toxicity. A clear mechanistic rationale supported by the literature relating to either electron or proton cycling was required for a fragment to be defined as a chemotype. Nine new chemotypes were defined, which were able to identify 89 of the 171 reported mitochondrial toxicants. Seven of the nine chemotypes related to disruption of normal mitochondrial function via acting as alternative electron acceptors. The remaining

two chemotypes corresponded to uncoupling of oxidative phosphorylation by proton cycling. Work is ongoing to further refine and extend the chemotypes, using the structural motifs, and relevant physico-chemical properties, that have been identified as inducing mitochondrial toxicity. The developed chemotypes could then be implemented into KNIME nodes or the ChemoTyper software. The chemotypes are intended to be used for grouping chemicals into categories within the AOP paradigm, to allow for the prediction of organ-level toxicity via read-across.

COSMOS is also contributing to the case study on the use of biomarkers to substantiate the read-across prediction (see section 3.4.3), investigating whether biomarkers from '-omics' investigations can increase the mechanistic knowledge on the individual compounds. **COSMOS** is contributing to chemical selection, mining data in COSMOS DB and other resources as well as helping in the identification of similar compounds. Read-across approaches are generally a focus of the **COSMOS** project modelling work. Moreover, ongoing work within **COSMOS** on toxicokinetics will be able to support the case study, e.g. by applying physiologically-based toxicokinetic (PBTK) models developed to the analogue compounds identified.

Similarly, **COSMOS** is contributing to the case study on mode-of-action based classification models for repeated dose liver toxicity (see section 3.4.6), aimed at distinguishing between potential hepatotoxicants and non-hepatotoxicants related to cholestasis, fibrosis and steatosis. The chemicals selected for the case study include substances for which PBTK models have been developed for *in vitro* to *in vivo* extrapolation (IVIVE) studies within **COSMOS**. Further synergies with on-going work exist regarding the development of liver toxicity structural alerts and data mining of COSMOS oRepeatTox DB to identify chemotypes relevant for liver toxicity (steatosis, steatohepatitis, fibrosis) of cosmetics-related chemicals.

COSMOS is also intensively involved in the cross-cluster read-across case study on the application level (see section 3.5.1), in particular in the investigations to identify the pairs and categories of analogues to be taken forward for the case study and for possible generation of *in vitro* data within the **SEURAT-1** Research Initiative supporting the evaluation through read-across for risk assessment. To this end COSMOS DB and other resources were mined for available repeated dose toxicity data and similarity analyses were carried out to match analogues in a list of initially proposed chemicals and within ToxCast data.

COSMOS will also contribute to the *ab initio* case study on integrating the Level 2 case study results for a quantitative mechanistic safety assessment, for example with the cheminformatics approaches such as the cosmetics TTC and chemical grouping to the proposed framework-workflow. Exposure considerations and the internal concentration in *in vitro* measurements are crucial aspects of the case study. **COSMOS** will be able to contribute the extrapolation of doses to free concentrations with the PBPK models and IVIVE approaches developed in the project.

4.6.3 Selected Highlight I: Public Release of the COSMOS Database Ver 1.0

Introduction

The management and sharing of chemical, biological and toxicity data in a flexible and sustainable manner play a central role within the **COSMOS** project. The TTC concept and (Q)SARs are two important approaches to perform risk assessment and predict the toxicological effects of chemical compounds. They depend on the availability of high quality toxicological data, linked to chemical structures, which should be openly available.

However, toxicological data for most mammalian endpoints and repeated dose toxicity in particular are scarce (*Cronin, 2009*). In particular there was no single inventory of cosmetics ingredients incorporating high quality and validated chemical structures. Such an inventory is required to appreciate the chemical space of cosmetic materials and to enable chemical grouping and modelling. This area is further complicated by the lack of open databases and current confusion over ontology for toxicological endpoints in the field of repeated dose systemic toxicity testing.

These needs are placed in the context of computational modelling as a key focus in 21st Century Toxicology and the requirements for modern toxicological science.

Approach

The COSMOS Database (DB) is a chemo-centric system which provides chemical and toxicological data to support the data needs of **COSMOS** and the projects of the **SEURAT-1** Research Initiative, as well as safety assessors in public and private organisations.

COSMOS DB is a relational database, built on top of publicly available open source software which makes it transparent and eases maintenance. The main datum is the chemical compound with its chemical structure and annotations. COSMOS DB stores these data and provides a number of specialised chemistry searches including similarity, substructure and full structure search to the end user. COSMOS DB has been designed and developed based on open-source chemistry development kit (RDKit) database technology (PostgreSQL database). It makes its content available to the end user via a user-friendly web interface.

COSMOS DB integrates data from various sources into a unified data model. Chemistry data have been collected from various sources, with special emphasis on cosmetics-related chemicals. The backbone of COSMOS DB is the COSMOS Cosmetics Inventory, a compilation of cosmetics-related substances from the European Union Inventory of 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 associated with 66 unique use functions.

The data model has been developed to accommodate full-dose level toxicity information, as well as dermal absorption/skin permeability data, and metabolism information. The US FDA (Food and Drug Administration) direct food additives and colourants PAFA database has been imported and a new database, oRepeatTox DB, has been constructed from oral toxicity data harvested by **COSMOS** partners for cosmetics-related chemicals. Sources include the US FDA OFAS (food contact substances) databases, US EPA (Environmental Protection Agency) ToxRefDB, National Toxicology Pogramme (NTP) reports, and European Commission SCCS (Scientific Committee on Consumer Safety) opinions and the scientific literature. In addition, further data were donated by a Cosmetics Europe member and **COSMOS** partners.

Results: Data Quality, Searching Functionalities and Data Mining

COSMOS DB ver1.0 (see *Figure 4.18*) was released to the public on 10 December 2013 with the URL: http://cosmosdb.cosmostox.eu. This was after performing a final usability testing with **COSMOS** consortium members and external parties which had shown interest in COSMOS DB, including Cosmetics Europe members, to insure the intuitive use of the database with minimum user training.

The public released COSMOS DB ver1.0 contains over 80,000 chemical records with more than 44,000 unique structures as well as more than 12,000 toxicity studies across 27 endpoints for over 1,600 compounds, in total.

Figure 4.18 *COSMOS DB entry page for database searching and links to COSMOS Space and ToxBank.*

Data Quality: Quality control and assurance of COSMOS DB were performed for both the chemical and toxicological data content, based on the data governance concept adopted by COSMOS DB. Within this process, two data quality issues have been addressed: (i) data record reliability (accuracy and completeness) and (ii) data acceptance.

For data accuracy, a web-based Data Entry System (DES) was prototyped in the internal COSMOS DB, for partners only, with a comprehensive controlled vocabulary set such that entry errors can be minimised. The quality control (QC) process focuses on examining the accuracy of the database content checked against predefined standards. In particular the connection tables, registry numbers and names were verified, as well as chemical structure and compound annotations. For toxicity data, a sample of 2722 records was verified for correctness and completeness against the original data sources.

The data completeness aspect of the record reliability has been addressed by establishing the COSMOS MINIS (MINImum Study) Criteria for study inclusion. The COSMOS MINIS Criteria were derived from in depth evaluation of the three regulatory guidelines for testing of repeated-dose toxicity studies – OECD, US FDA and US EPA – and implemented in the COSMOS DB DES. They represent the set of required and recommended experimental parameters to be considered to 'meet the criteria'. The data record reliability is objectively rated by a **COSMOS** score for 'meeting the guidelines', 'meeting the COSMOS MINIS criteria', 'not meeting the COSMOS MINIS criteria', or 'not usable'.

The data acceptance aspect of the data quality has been addressed by conducting data content QC in collaboration with external experts in the toxicology and risk assessment field. The acceptance for study inclusion data has been captured as Klimisch scores in the oRepeatTox DB.

The MINIS criteria and **COSMOS** scores are implemented in COSMOS DB. Data record reliability can be calculated algorithmically by the COSMOS DB DES, indicating the degree of study completeness during the data entry stage. The Klimisch scores, on the other hand, are assigned manually by the toxicologist experts.

The corrected information for the checked compounds was implemented in the production database to improve its reliability. Statistics of errors were reported (see section 4.10.3.3).

COSMOS DB Searching Functionalities: COSMOS DB supports data retrieval via a user-friendly web interface which allows querying by the chemical, the toxicological or both types of data. The chemical search can be carried out by name, registry numbers or other identifiers (e.g., COSMOS IDs, DSSTox IDs) provided for a single structure or for a multiple chemicals list, as well as by structure (sketched or as SMILES string). Exact substructure and similarity structure search types are possible. Similarity searching was implemented using the RDKit fingerprints (*Landrum, 2012*) and pair-wise Tanimoto coefficients.

The scope of the toxicological queries can be defined in detail by the users with respect to the endpoint (study type) and endpoint-specific study parameters (e.g., species, strain, sex, route of administration, cells/cell lines, test calls, target sites). The toxicological data of interest can be retrieved for all relevant compounds included in the database (if no query chemicals are defined) or just for the specified compounds of interest. Screenshots of a query page of such a composite search combining structure and study information and an example of a database retrieval of toxicity data are shown in *Figures 4.56-4.58* in section 4.10.3.3.

Data Mining of COSMOS DB: COSMOS DB allows data mining not only to search for toxicity data but also to support grouping approaches within an AOP framework. In one case study, in order to identify the alerting substructural fragments relevant for liver toxicity – steatosis, steatohepatitis, fibrosis – of cosmetics-related chemicals, the COSMOS oRepeatTox DB was subjected to systematic, ontology-based data mining. The basis ontology set consists of hierarchically structured toxicity effects: phenotypic effects observed at the organism level and more specific effects observed at subsequent (lower-level) sites: Organs/Systems-Segments/Tissues-Cells/Organelles. The controlled vocabulary organised in this way enables mechanistic mining of data included in the COSMOS oRepeatTox DB.

COSMOS oRepeatTox DB contains 228 cosmetics-related chemicals, for which 340 oral studies (sub-chronic, chronic, studies about reproductive and developmental toxicity, short-term studies with >28 days) were harvested from available regulatory and literature sources (such as SCCS opinions, NTP reports) as well as primary literature publications. The studies cover the range of assays and measurements for rat (31 chronic, 200 short-term/sub-chronic, 31 studies about reproductive and developmental toxicity), mouse (13 chronic, 19 short-term/sub-chronic, 3 studies about reproductive and developmental toxicity) and dog (7 chronic, 7 short-term/sub-chronic studies). They include in-life parameters (body weight gain, clinical signs, clinical chemistry, hematology, mortality, urinalysis), 'after sacrifice' investigations (organ weight, necropsy, histopathology), parental, reproductive and developmental (pre-natal) toxicity. Toxic effects observed at each level are described in terms of their severity, direction (decrease/increase/no change), toxicological and statistical significance. Histopathological lesions are recorded for a wide range of target organs, including liver, kidney, heart, lung, stomach and forestomach, spleen, and thyroid gland.

Data mining the toxicity effects captured in the oRepeatTox DB disclosed that over 25% of cosmetics-related chemicals in this database are associated with lipid deposition, fatty acid changes, cytoplasmic vacuolisation, cellular infiltration and inflammation in hepatocytes leading to fibrosis. These phenotypic effects and morphological changes are involved in steatosis, steatohepatitis, and fibrosis at various liver sites. From the database of 228 chemicals, 59 structures were found to be associated with such 'effects' terms in the database. Application of ToxPrint chemotypes (http://toxprint.org) to these structures using the publicly available

free software tool ChemoTyper (http://chemotyper.org) allowed the identification of generic chemotypes common to these structures. The chemotypes are defined as structural fragments encoded with atom/bond properties and can be further refined by precise annotation to carry specific biological activity information (e.g., phenotypic effects). For the example of 'liver', the first set of common general chemotypes are considered 'proto-steatosis-chemotypes', which then are further annotated with partial charges and shapes to the final set of 'steatosis-chemotypes' (i.e., steatosis alerts). The set of alerting chemotypes for liver steatosis/steatohepatitis/fibrosis includes alcohols (short chain), diols, glycol ethers (and repeating oxirane ether), aminophenols, tertiary amines and tertiary aromatic amines, polychlorinated short alkanes, halogenated amines, Michael acceptors, and hydrophobic flat (or aromatic) rings with flexible extenders. Identification of liver effects-specific chemotypes is the initial step in developing the liver toxicants categories and provides a way to investigate molecular pathways relevant to toxicological mechanisms.

4.6.4 Selected Highlight II: Evaluation of the Applicability of the Threshold of Toxicological Concern Approach to Cosmetics

Introduction

In development of alternatives to animal testing, the Threshold of Toxicological Concern (TTC) approach can serve as one of the practical safety assessment tools for chemicals for which no *in vivo* testing results are available (see also section 2.3). It is a risk assessment paradigm that establishes a human exposure threshold value for chemicals, below which there is a low probability of an appreciable risk to human health. This approach was inspired by and can be considered an extension of the Threshold Of Regulation (TOR), although not equivalent, adopted by the US Food and Drug Administration for substances used in food-contact articles (*FDA, 1995*). The original TTC concept used a single threshold for all chemicals, based on the conservative assumption that an untested chemical could pose a cancer hazard. It was subsequently expanded to include non-cancer endpoints by *Munro et al. (1996)*.

One of the goals of the **COSMOS** project is to extend the current TTC approach to cosmetic ingredients. The current non-cancer TTC assessment is based on the dataset used by *Munro et al. (1996)*, which contains 613 diverse tested chemicals and their 'No Observed Effect Level' (NOEL) values from oral repeat dose toxicity studies. Transforming the data to chronic 'No Observed Adverse Effect Level' (NOAELs), *Munro et al. (1996)* identified the 5th percentile of the cumulative distribution for each Cramer Class (*Cramer et al., 1978*) and devised the current thresholds. Recently, the European Food Safety Authority (*EFSA, 2012*) re-evaluated the use of TTC for foods. In addition, the European Commission's non-food Scientific Committees (SCCS, SCHER and SCENIHR) in 2012 also published an opinion on the use of TTC for chemicals in cosmetics products (*SCCS/SCHER/SCENIHR, 2012*). The Scientific Committees stated that the TTC approach is scientifically acceptable, whilst noting some

concerns, including that all risk assessment approaches have some degree of uncertainty, many complex chemical structures are not adequately represented in currently available databases, and that there is limited knowledge of effects due to dermal and inhalational exposure routes that are more common for consumer products.

Application of the TTC approach to ingredients used in personal care and household products was evaluated by *Blackburn et al. (2005)*, who used data from Procter & Gamble to assemble a repeat dose toxicity database of 248 substances, 29 of which were represented in the Munro database (*Munro et al., 1996*). Of the 219 novel substances, 145 could be assigned to a specific Cramer class but suitable NOAELs for comparison with the Munro database were only identified for 45 substances (21, 2, and 21 of which fell in to Cramer classes I, II and III, respectively). The highest and mean NOAELs were similar for the two datasets, but the lowest NOAELs were lower in the Munro database. The authors concluded that these results support the use of the TTC concept for ingredients in personal and household care consumer products.

COSMOS addresses the key issues in applying the current approach to cosmetic ingredients, including

- Applicability of the chemical domain of the non-cancer database (*Blackburn et al., 2005*);
- Applicability of the Cramer Decision Tree for protectiveness (*Blackburn et al., 2005*);
- Extrapolation of the oral-to-dermal route exposures (*Kroes et al., 2007*).

Approach

For these three issues, the following approaches have been adopted: (i) the chemical space of the current Munro database may need to be expanded with addition of new data to sufficiently cover the cosmetic ingredients and other chemicals found in cosmetics products; (ii) the Cramer Decision Trees may be modified to reflect biological pathways or mode-of-action categories; (iii) the need for target organ dose extrapolation due to oral-to-dermal exposure differences may be addressed by incorporating absorption, distribution, metabolism, and excretion (ADME) knowledge.

To this end, **COSMOS** has established two Expert Groups (EGs) with ILSI-EU: Expert Group 1 for the development of criteria to be applied in the extension of the current TTC approach to cosmetics-related chemicals and Expert Group 2 for the evaluation of oral-to-dermal extrapolation.

The basis for the planned evaluation of the TTC approach to cosmetics-related chemicals has been the compilation of a new oral repeated dose toxicity database, oRepeatTox DB (included

in COSMOS DB), to use as a resource to build the new COSMOS non-cancer TTC database of NO(A)ELs, enriched with cosmetics ingredients.

For the assessment of the safety of dermal exposure through extrapolation from oral toxicity data by applying ADME knowledge, taking into consideration exposure scenarios and bioavailability issues relevant to cosmetics, some difficult outstanding issues had to be addressed. First, the existing dermal absorption/skin permeability databases did not contain enough cosmetics-related chemicals. Therefore, a skin absorption database enriched with cosmetics-related chemicals was build. Moreover, understanding the differences in bioavailability between oral and dermal exposures also requires knowledge and data of metabolism and other gastrointestinal specific factors. *Kroes et al. (2007)* evaluated 58 Cramer class III chemicals with NOAEL values of 1 mg/kg or less. They evaluated the data to determine whether the oral-route toxicity could be used for predicting dermal-route toxicity more accurately by including metabolism in the liver (systemic) and first pass oral versus dermal metabolism. Some of the chemicals for which metabolism was considered were highly toxic, such as pesticides, and did not overlap with the chemicals in the COSMOS Cosmetics Inventory. The cosmetics-related chemicals in the COSMOS TTC database are expected to have in general lower toxicity and therefore higher NOAEL values than chemicals included in the analysis of *Kroes et al.* (*2007*), which will lead to a greater safety margin of exposure levels.

Results

A general oral repeat-dose toxicity database is the key to this process when compiling the appropriate studies to be used in construction of the TTC dataset. These studies provide underlying data that can be evaluated for appropriate NOAEL and LOAEL decisions. It is also important to house the NOAEL/LOAEL values in a separate simpler database with critical effects as well as the sources of the decisions and their rationale. Hence the approach was to build a database with reliability scores for methods and results.

The oRepeatTox DB, a subset of COSMOS DB, has been compiled from various database sources including US FDA PAFA and CERES databases, US EPA ToxRefDB and EU SCCS (Scientific Committee of Consumer Safety) opinions. Currently, the resulting collection includes over 1,000 compounds covering toxicity studies with target organ effects from sub-acute (duration ≥ 28days), sub-chronic, chronic, carcinogenicity (non-neoplastic lesions), reproductive-developmental toxicity, neurotoxicity, and immunology studies. Species were limited to rat/mouse for target organs, rat/mouse/rabbit for reproductive-developmental toxicity, and dog/monkeys for all studies except neoplastic effects.

The ILSI-EU COSMOS TTC Expert Group 1 identified a prioritised list for more detailed review of data in order to support the data-acceptance reliability. To this end, two types of data were identified for in-depth review: (i) studies whose NOAEL values fall in the lower 10% of the

distribution for each Cramer Class; (ii) studies whose NOAEL values differ from one source to another by more than two orders of magnitude. The chemicals in this prioritised list were selected for re-harvesting to check/update/correct the records in the oRepeatTox DB.

A set of study selection criteria, summarised in *Table 4.9*, were applied to extract the COSMOS TTC database from the oRepeatTox DB.

Table 4.9 *Study inclusion criteria and exceptions in defining databases and dataset.*

Parameters	Rules for TTC dataset	Exceptions for oRepeatTox DB	Exceptions for TTC database
Study type	sub-chronic, chronic, carcinogenicity (non-neoplastic), reproductive, developmental, neurotoxicity, immunology	neoplastic lesions of carcinogenicity studies are not included	reproductive-developmental studies only for critical systemic effects
Species	rat and mouse (all studies), monkey and dog (all studies)	rabbit (reproductive and developmental toxicity)	rabbit (reproductive and developmental toxicity)
Duration	≥ 28 days for sub-acute and sub-chronic	For reproductive and developmental or multi-generation studies, simple duration days are not applied	For reproductive developmental or multi-generation studies, simple duration days are not applied.
Route of exposure	dietary, drinking water, gavage (or intubation)	no exceptions	no exceptions
Dose levels and range	- single dose studies are not used. - dose separations (low, mid, high) are reasonable.	All studies with dose level and regimen information are included	no exceptions
Effects	systemic effects	all effects are recorded	no exceptions
Reference	traceable citation	regulatory submissions	regulatory submissions

The COSMOS non-cancer TTC database includes over 500 cosmetics-related chemicals and impurities as well as NOAEL/LOAEL values from studies meeting the criteria defined by ILSI-EU Expert Group 1.

The data record quality of the relevant TTC database has been rated by COSMOS MINIS (minimum study) criteria and the data acceptance has been addressed by a group of ILSI-EU experts. The second study review session (ILSI-EU QC2) considered 51 cosmetics-related chemicals. The selections were made for the studies whose NOAEL values appear in the lowest 10th percentile of the distribution for each Cramer Class and the compounds whose data vary widely across the different data sources. The data acceptance of the studies used in the review sessions was assigned by experts.

The chemical space of the COSMOS non-cancer TTC database includes cosmetics-related chemicals. A greater fraction of chemicals in the COSMOS TTC database belongs to Cramer Class I than in the Munro database (in comparison, 73 % of Munro is Cramer Class III). COSMOS TTC and Munro are represented broadly by similar chemotypes although there are some differences in frequencies; for example, organosilicons (**COSMOS** only), chemotypes representing hair dyes (more prevalent in **COSMOS**), organohalides (more prevalent in Munro), and longer aliphatic (C≥8) chains (more prevalent in **COSMOS**). Profiles of physico-chemical properties distinguish the COSMOS non-cancer TTC database from the Munro database. Cosmetics-related chemicals tend to have lower LogP and higher water solubility. A comparison of the chemical classes in the COSMOS Cosmetics Inventory, the COSMOS TTC dataset and in the Munro dataset is shown in *Figure 4.19*.

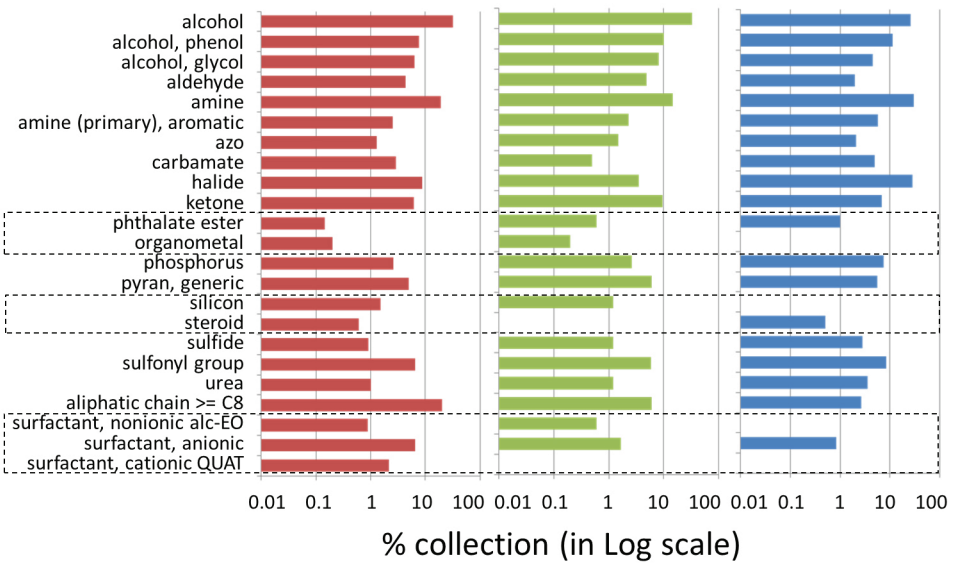

Figure 4.19 *Structural classes (chemotypes) in the COSMOS Cosmetics Inventory (red), in the COSMOS TTC dataset (green) and in the Munro dataset (blue).*

The COSMOS non-cancer TTC database has been evaluated for inclusion/exclusion of certain compound classes. These compound classes include biologically active ingredients, chemicals banned in the EU (Annex II of EC regulation), hair dyes, organophosphates, and genotoxicants/DNA binders.

The COSMOS TTC dataset is extracted from this COSMOS TTC database using NOAEL/LOAEL selection criteria (see *Table 4.9*). The minimum NOAEL value is selected from among several data sources. When NOAEL values are not available for a given compound, a minimum

LOAEL value was taken for the test substance.

In summary, from the oral toxicity data to the TTC dataset, two new databases and a dataset have been established in the COSMOS project. The curation process used to build oRepeatTox DB, COSMOS TTC database, and the COSMOS TTC dataset is depicted in *Figure 4.20*.

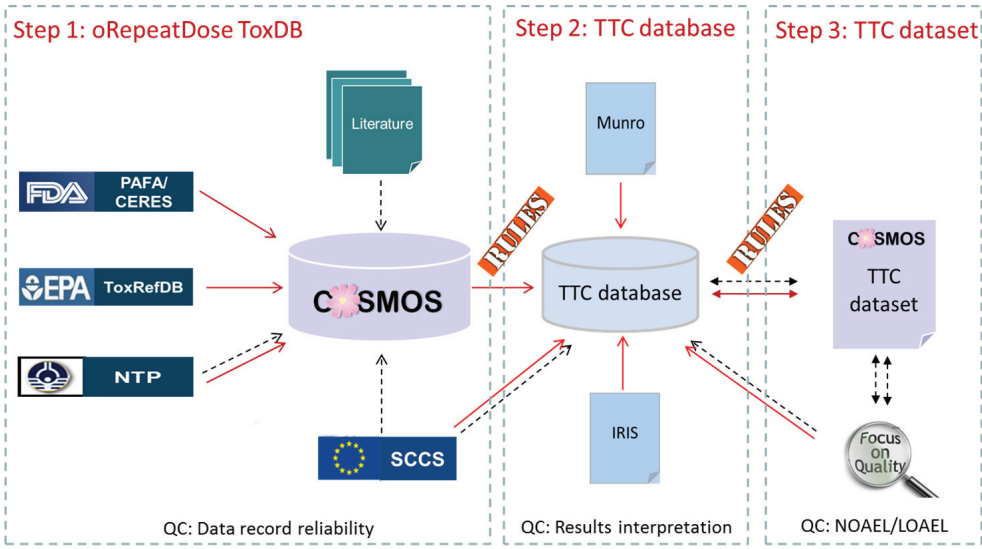

Figure 4.20 *Data curation process from oral toxicity data to TTC dataset. Red solid arrow indicates automated process; black dotted arrow indicates manual process.*

The COSMOS skin permeability database enriched with cosmetics-related chemicals was created to support the oral-to-dermal extrapolation efforts to determine safety following dermal exposure, as well as models for predicting dermal absorption. It has been curated from three sources: (i) the existing EDETOX database (http://edetox.ncl.ac.uk); (ii) data donated by Dr Taravat Chafourian, Medway School of Pharmacy (Samaras et al., 2012); (iii) **COSMOS** harvesting.

An essential issue was the accuracy and quality-control of the dataset in order to allow the building of reliable models based on it. This would increase confidence upon predictions made from the models for which a clear applicability domain should be determined. The COSMOS skin permeability database has attempted to address the concerns over the accuracy of data by instigating a quality control and checking procedure, with a particular emphasis on the published data after the EDETOX database has been established.

A tiered decision-tree approach has been developed by ILSI-EU Expert Group 2 as a guide to estimate systemic bioavailability following dermal exposure to cosmetics when applying the oral TTC in the absence of toxicity data (*Williams et al., 2014*). The decision tree is based

on estimated usage (i.e., skin exposure) and dermal absorption derived from a prediction of maximal flux to estimate actual absorption. The influence of differences in metabolism and efflux transport in skin and gastrointestinal tract on bioavailability was also considered.

Use cases have been developed to evaluate the applicability of the decision tree to assess the safety of dermal exposure, based on the TTC concept. The decision tree was evaluated with exposure scenarios for five cosmetic molecules: methylisothiazolinone, diethylphthalate, resorcinol, butyl paraben and quercetin and the contaminant dioxane.

In summary, **COSMOS** has considered how to apply the TTC concept taking into account chemical structure, Cramer class and predictions or measurements of skin permeability and dermal systemic dose.

4.6.5 Innovation

Public Release of the COSMOS Database Ver1.0: The public release of the COSMOS DB ver1.0 has a broad socio-economic impact. This database launch greatly improves the data availability for repeated dose toxicity data of cosmetics-related chemicals. Entities impacted by this work include regulatory agencies, cosmetics industry, research institutes, universities, small/medium enterprises and NGOs. For example, COSMOS DB has been made available to US CIR (Cosmetics Ingredients Review) and NITE Japan (National Institute of Technology and Evaluation) to be imported to HESS (Hazard Evaluation Support System).

COSMOS DB is not static. The **COSMOS** consortium plans to maintain and update the content and the software on a regular basis. The next updates will see the COSMOS DB content expanded with the integration of a dermal absorption (skin penetration) dataset as well as the highly curated COSMOS database for non-cancer TTC.

COSMOS DB is a comprehensive and useful source not only to support predictive toxicity modelling and safety assessment, but also lending itself to be mined for information feeding into mechanistic considerations and building Adverse Outcome Pathways.

Evaluation of the Applicability of the Threshold of Toxicological Concern Approach to Cosmetics: There is increased interest in the prospect of broadening the use of the TTC concept in the regulatory context, as evidenced by the number of comprehensive reviews of the topic recently undertaken by various regulatory agencies and the involvement of regulators in several recent symposia and fora that pertain to TTC. The appeal of TTC to regulators is that it holds the potential to provide a pragmatic, transparent, consistent and scientifically sound approach to prioritisation, allowing for the allocation of finite resources to the testing and evaluation of those substances with the greatest potential to pose risks to human health. Expanding the TTC approach to the risk assessment of cosmetics-related ingredients, for which chemical-

specific toxicity data are lacking, may thus have an enormous impact on risk assessment.

The new COSMOS TTC database for the application and extension of the TTC approach will be fully transparent and open so that all decisions taken can be retraced and understood. It is planned to make the COSMOS TTC dataset available in a form that stakeholders can use and also complement it, and will be able to re-perform the NO(A)EL analyses, if they wish so. The approach of the COSMOS ILSI-EU TTC Expert Groups will also be described in detail in major publication(s) about the new TTC dataset and the oral-to-dermal-extrapolation approach and decision tree adopted.

Among the strategies to address the application of TTC values derived from oral data to use cases relevant to cosmetics, a tiered approach has been elaborated for the assessment of the chemicals' bioavailability, which takes into account the absorption/permeability via dermal or oral routes as well as metabolism differences between skin and liver. This provides novel models indicating possible systemic bioavailability following dermal or oral absorption.

4.6.6 Cross-Cluster Cooperation

COSMOS has interacted with the other projects of the **SEURAT-1** Research Initiative in many ways, starting with the involvement of some partners in several projects.

Cross-cutting activities which span across **COSMOS** work packages include, for example, the mode-of-action approaches leading to Adverse Outcome Pathways (AOPs) and the oral-to-dermal extrapolation encompassing metabolism and bioavailability considerations. In particular, the work leading to the development of AOPs has been taken up at the **SEURAT-1** level and is forming the basis of several cluster level case studies. **COSMOS** partners are involved in the Mode-of-Action and Biokinetics Working Groups, the latter being co-lead by **COSMOS** partner 'French National Institute for Industrial Environment and Risk (INERIS)'. The overarching cross-cutting activities regarding AOPs are indeed a major theme within **SEURAT-1** with **COSMOS** efforts on mitochondrial toxicity being highlighted. **COSMOS** has also supported **SEURAT-1** case studies on read-across through the searching of COSMOS DB for data, as well as other sources, and the provision of chemoinformatics support for similarity analysis. Similarly, **COSMOS** will contribute to the case study on *ab initio* safety assessment.

COSMOS is also involved in the **SEURAT-1** Training Task Force and, for example, actively contributed to the programme of the **SEURAT-1** Summer School in The Netherlands in June 2014 through a number of sessions and lectures. It is also leading the way with the provision of webinars (e.g. for COSMOS DB).

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

COSMOS compiled the Cosmetics Inventory v1.0 as the first comprehensive compilation of

cosmetics-related substances. As well as being embedded within COSMOS DB, which has been made publicly available, it is provided as a standalone dataset and can thus be shared with the **SEURAT-1** Research Initiative. Throughout the third year **COSMOS** interacted with ToxBank to establish the interoperability between COSMOS DB and the ToxBank data warehouse.

The COSMOS work on Physiologically-Based PharmacoKinetic (PBPK) models and *in vitro* to *in vivo* extrapolation led to the development of a case study about multi-scale modelling with acetaminophen, in single and multiple dose situations in corporation with, and using data from the **SEURAT-1** DETECTIVE project. Another line of work includes coupling, with an *in silico* model of liver, the internal metabolism of the hepatocytes (data from the **SEURAT-1** NOTOX project) with a simple 3D model of the liver and predict toxic effects distributed in space and time inside the organ.

A first version of the cell-based assay model using an open source platform (KNIME/R) has been completed. This model has been made available to interested partners of the **SEURAT-1** Research Initiative so they can characterise, analyse and simulate the dynamics of their cell-based assays experiments. Moreover, a simple PBPK model coded in R has been provided. This model permits a prediction of the time-course of the substance concentration in different organs for a given exposure scenario (unique dose or repeated doses). It can combine three routes of exposure (dermal, oral and inhalation), currently with a focus on the liver. It is expected that the set of complete models will allow improving the results of *in vitro – in vivo* extrapolation. **COSMOS** Partner INERIS has held workshops and dissemination events for biokinetics modelling.

4.6.7 Expected Progress within the Fourth 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**. COSMOS DB ver1.0 has been succesfully made publicly available as a resource to retrieve and mine toxicological information and data in December 2013. COSMOS DB will be developed further in the fourth year of the project and updates will be released with the inclusion of further data on e.g. skin permeability. This will lead to an comprehensive open database by the end of the project. Furthermore COSMOS Space, which facilitates user interaction and sharing of predictive toxicology resources, will further be populated and promoted.

COSMOS has compiled a database for Threshold of Toxicological Concern (TTC) analysis and this will make the data transparent for any possible further work. Specifically, in the fourth year, **COSMOS** will deliver the quality-controlled COSMOS TTC dataset of repeat dose NO(A)EL values, also documenting the study inclusion criteria and rules used to determine NO(A)ELs based on data from various sources. Furthermore, a set of bioavailability data

including skin penetration and Caco-2 cell permeability will be provided. The COSMOS TTC dataset can provide the basis for the thresholds for cosmetics ingredients, considering also the oral-to-dermal extrapolation. To extrapolate data from the oral to dermal route (relevant for many cosmetics) a tiered workflow will be provided taking into account bioavailability via the different routes i.e. differences in uptake and metabolism. Within the scope of the **SEURAT-1** Research Initiative, **COSMOS** will deploy a software tool of the TTC database and a workflow implemented in KNIME.

The **COSMOS** project will continue to embrace new ways of thinking such as the application of molecular modelling techniques to toxicity prediction and the development of Adverse Outcome Pathways (AOPs). Specifically the development of chemotypes for AOPs relevant to organ level toxicity will be pursued further in the fourth year. Chemotypes extend and expand the structural alert concept by inclusion of other relevant physico-chemical properties. The dataset containing physico-chemical properties, structural information, and *in vivo* data available from the COSMOS Database will be used to compare different approaches such as read-across, grouping and QSAR models. These *in silico* methods will also be employed to refine structural categories for toxicity prediction. Furthermore, the information on the biological profile of the chemicals will be considered by similarity analysis. QSAR models and expert systems predicting the chronic toxicity endpoints will be searched for suitable groups of the chemicals of the COSMOS Cosmetics Inventory. The key part for **COSMOS** is the definition of the molecular initiating event and the possibility of using this for chemical grouping and read-across and this will link with the broader work within **SEURAT-1** to develop AOPs. The effective extrapolation of the effects of an *in vitro* concentration into a *in vivo* dose is also an important goal of **COSMOS**. The fourth year of the project will see the development of more descriptive approaches using a toxicity pathways and mode-of-action framework, systems biology models at molecular level. Specifically, molecular metabolic and control networks for selected cell lines will be developed.

All activities in **COSMOS** will be supported by the KNIME software, resulting in openly available and transparent workflows. With the increasing use of the KNIME Server prototype, additional functionality or usage improvements will be required in the next phase of the project.

During the fourth year **COSMOS** will continue to actively contribute to the **SEURAT-1** Case Studies.

4.6.8 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 for the development of alternatives. **COSMOS** will provide an open database, both in terms of the structure and implementation but also the data contained. COSMOS DB Ver1.0 has already been made publicly available and 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.

COSMOS will develop Threshold of Toxicological Concern (TTC) approaches better suited to classes of cosmetics compounds. Specifically, **COSMOS** will provide a new database for the application and extension of the TTC approach to the cosmetics area. The new non-cancer TTC database, developed in collaboration with expert toxicologists, will be transparent, open and fully documented incorporating recommendations for appropriate use. A tiered workflow taking into account bioavailability via the different routes will be provided to extrapolate data from the oral to dermal route. TTC is a pragmatic method recommended by EU EFSA and SCCS in safety/risk assessment of chemicals found in food, cosmetics or consumer products. Hence, the new TTC database is anticipated to have broad impact on the cosmetics industry well beyond the **SEURAT-1** community.

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 Adverse Outcome Pathways (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. The mechanistic considerations provide a cornerstone for the cross-cutting activities within the **SEURAT-1** Research Initiative and beyond. Work within **COSMOS** can be used to inform AOP development within the framework of current OECD projects in this area.

Models for toxicodynamics and toxicokinetics are being 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 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.

In summary, the research undertaken in the **COSMOS** project will ultimately support the area of computational modelling as it is being implemented in the vision of 21st Century Toxicology. Overall this will enable more relevant and reliable information relating to human safety to be obtained; it will contribute to the reduction of animals for toxicological assessment; and it will assist in the development of cheaper and greener products.

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Awards

The QSAR and Molecular Modelling Group, Liverpool John Moores University, England received the 2013 Lush Science Prize

The group received the Lush Science Price for developing computational alternatives to animal testing to predict the effects of chemicals. The Lush prize is an annual prize fund for researchers working in the alternatives to animal testing field, focusing on consumer products and ingredients, and is funded by Lush cosmetics in the UK, a company committed to the non-animal test methods for all of their products, and Ethical Consumer magazine.

Dr. Steve Enoch was jointly awarded the 2013 Lush prize for science for the work of the QSAR and Molecular Modelling group at Liverpool John Moores University to develop *in silico* methods for the non-animal risk assessment of skin and respiratory sensitisation. The award was made for notable contributions to the field of predictive toxicology focussing around efforts on the development of computational methods applicable to cosmetics ingredients.

Poster Awards

Hristozov, D., Jeliazkova, N., Kleinoeder, T., Lan, Y., Meinl, T., Miller, S., Neagu, D., Schwab, C.H., Richarz, A.-N., Hardy, B., Cronin, M.T.D., Yang, C. (2014): COSMOS Database: Public availability of repeated dose toxicity data and collaborative interoperability with the ToxBank data warehouse supporting integrated data analysis. Poster award at the SEURAT-1 4th Annual Meeting 2014, 5–6 February 2014, Barcelona.

Kovarich, S., Bassan, A., Cronin, M.T.D., Fioravanzo, E., Manelfi, C., Worth, A.P., Yang, C. (2013): Molecular Modelling to Predict and Understand Chemical Toxicity in the AOP framework – Case Study: MoA from LXR Activation to Liver Steatosis. Poster award at the SEURAT-1 3rd Annual Meeting 2013, 6–7 March 2013, Lisbon.

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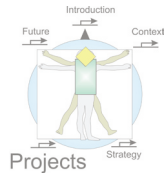
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4.7 NOTOX: Predicting Long-term Toxic Effects using Computer Models based on Systems Characterization of Organotypic Cultures

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4.7.1 Introduction and Objectives

Validated alternative assessment methods for long-term systemic toxicity are urgently required to cope with the complete ban (enforced from 11 March 2013) on animal testing of cosmetic products in Europe. 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 (humans) is not possible, human organotypic cultures are applied to permit reproducible and transferrable testing of the highest possible relevance. Multi-scale models will 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 the liver plays a central role in metabolism, in both its inherent and xenobiotic conversion functions, we selected hepatic cultures for the **NOTOX** project. As human hepatic cells derived from stem cells are not yet readily available with sufficient functionality, we selected HepaRG, a hepatocarcinoma cell line, and primary human hepatocytes (PHH) for **NOTOX**. The HepaRG cell line has been shown to be closest to primary human cells in terms of the metabolism

of xenobiotics, 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 of selected test compounds over long timescales. The selected test compounds are of industrial relevance and have 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. The design of experiments will incorporate 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. Together with curated literature and genomic data, these toxicological data will be organised in a toxicological database (in cooperation with DETECTIVE, COSMOS and ToxBank).

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.

The effects of long-term exposure to test compounds as monitored and measured by the above-mentioned technologies will be analysed using bioinformatics methods. Data from databases, literature, experiments and simulations will be integrated through bioinformatics tools to create a knowledge base for quantitative understanding of adverse outcome pathways and regulatory networks at the molecular level. These data will provide the bases for prediction models. Large-scale modelling of regulatory and metabolic pathways will simulate toxic responses starting from molecular initiating events. Since such large-scale computational systems biology models often comprise a large set of equations and may include 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 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 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 (from various databases) and high-performance grid computing for identification of predictive endpoints.

➠ Development of predictive causal computer models aimed at entering pre-validation as guided by the integrative project (ToxBank) and as defined by ECVAM.

➠ 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.

4.7.2 Main Achievements and Challenges in the Third Year

Experimental System Biological Studies of Long-Term Toxic Effects Using Cellular Systems

3D HepaRG spheroid cultures (established in the first phase of project) were characterised in detail. These 3D HepaRG cultures were used in acute but also repeated dose toxicity studies. The 3D spheroid cultures are further extended to primary human and mouse hepatocytes (PHH and PMH, respectively). Moreover, co-cultures with non-parenchymal cells are also established for PHH and HepaRG cells.

Next, joint consortium-wide studies focused on AOPs were conducted. A pilot experiment applying acetaminophen was successfully carried out and the '-omics' data has been used for modelling (see below). This experiment triggered the development of a serum-free cultivation medium that allows for unbiased '-omics' analyses in further 2D and 3D large-scale experiments (details are given in section 4.7.3).

To plan long-term repeated-dose experiments, a pilot experiment was carried out for the determination of corresponding response curves for valproic acid, bosentan and

chlorpromazine. The next step was to characterise long-term toxicity with '-omics' analyses. We focused on valproic acid long-term repeated-dose toxicity (14 days) in 2D in a joint large-scale experiment. This was in the framework of the **SEURAT-1** case study on steatosis as the mode of action. The experiment was successfully carried out and data is being analysed (see section 4.7.4). A similar setup is repeated on a smaller scale with 3D HepaRG spheroids.

Epigenomic, Fluxomic, Metabolomic, Peptidomic, Proteomic and Transcriptomic Analyses

Various '-omics' analyses have been successfully completed on the pilot experiment on acetaminophen. Further analyses on the samples from the joint consortium-wide long-term repeated-dose experiment on valproic acid in 2D are in progress. Transcriptomics studies on long-term acetaminophen exposure of PHHs are been carried out. ^{13}C metabolic flux analysis was carried out in 2D using ^{13}C substrates.

Cryo-Light and Cryo-Electron Tomography with 3D-bioinformatic Analysis of Tomographic Data

A simple workflow in cryo-electron tomography (CET) and sub-volume averaging (SVA) was introduced, which allows improved high-resolution, reliable results to be obtained with little expert supervision. This technology can be used as a tool to link cell biology to structural biology, aiming for a more complete understanding of the physiological processes in the cell. Microscopic images with confocal, two-photon and light-sheet microscopy have been obtained. Electron microscope images of 3D HepaRG spheroids (control and treated) have been made and reconstruction of the 3D structure is in progress.

Large Multi-Scale Modelling of Long-Term Toxic Effects in Organotypic Cultures

The cellular acetaminophen acute toxicity model could be verified successfully against experimental time-series data gained in the first NOTOX pilot experiment. The model was modified so that it could be coupled with detailed 2D culture and 3D organoid models. This model was successfully applied, focusing on a multi-scale modelling approach combining cellular hepatic acute acetaminophen toxicity with 2D liver and whole body physiologically based pharmacokinetic (PBPK) modelling.

Addressing *in vitro–in vivo*-extrapolation (IVIVE), we adapted the previously published oral equivalent dose (OED) approach of *Wetmore et al. (2012)* for the estimation of safe/critical drug doses. Drug degradation and time-dependent response was measured *in vitro* and a virtual population was used to capture potential variability. Further, a PBPK model, which was

developed in-house and validated by **NOTOX** partner 'Insilico Biotechnology', was used for IVIVE and estimation of safe doses (NOAEL) by comparison with *in vitro* dose response data (see section 4.7.5).

Finally, 'CellSys' software was setup and used to run toxicity simulations using agent-based models. This model has been used to simulate acetaminophen toxicity in 3D spheroids (see section 4.7.5).

4.7.3 In vitro Cultivation of Organotypic HepaRG Cultures for Long-Term Repeated Dose Toxicity Studies

Serum-free Medium for Long-Term Cultivation of HepaRG cells

A system for long-term cultivation of liver cells has to maintain high liver specific functions as well as viability over time. In **NOTOX**, we investigated different cultivation conditions in order to find out which conditions are suitable for long-term cultivation of HepaRG cells. As serum hampers '-omics' analyses and interferes with the exposure to many compounds, we were particularly interested in serum-free conditions (SFM). Four different conditions without and one standard long-term medium with fetal bovine serum were tested. SFM1, 2 and 3 were supplemented with growth factors making up for the withdrawal of fetal bovine serum. SFM4 was used for cultivation without fetal bovine serum and growth factors. SFM1, 2 and 3 differed in respect to their DMSO concentrations (no DMSO, 0.5% DMSO and 1.8% DMSO, respectively; *Figure 4.21*). Investigation into the viability of 2D HepaRG cultures over time showed that HepaRG cells can be cultivated without addition of fetal bovine serum for at least 30 days, while maintaining viability in cells (SFM1 and SFM2; see *Figure 4.21*).

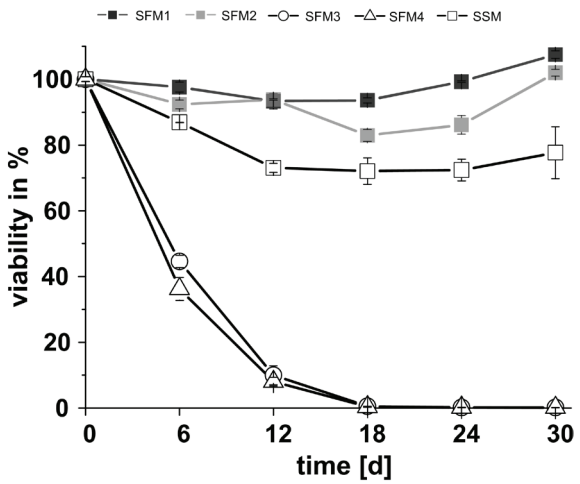


Figure 4.21 *Viability of HepaRG cells during 30 days cultivation upon maintenance in different media with daily medium renewal. SFM1 / 2 / 3 (Serum-free medium with growth factors and*

0% / 0.5% / 1.8% DMSO respectively), SFM4 (Serum-free medium with 1.8% DMSO) and SSM (Serum-supplemented medium with 1.8% DMSO). Viability was assessed using alamarBlue assay. Error bars indicate standard deviation (n = 3). Viability is given in percentage relative to HepaRG culture viability on day 0 (Klein et al., 2013).

At the same time, the cytochrome P450 (CYP) activity of HepaRG cells during long-term cultivation was monitored for those conditions keeping the cells viable over time (i.e., SFM1, 2 and SSM). Results are depicted in *Figure 4.22*. While activities for CYP1A2, CYP2C9 and CYP2D6 only differ slightly between the tested conditions (SFM2 and serum supplemented medium (SSM)), we found significantly higher activities for CYP2B6 and CYP2D6 when cells were cultured in SSM with 1.8% DMSO. For both, SFM1 and SFM2, CYP activities generally decreased from day 0 to day 30, however were still significantly high (around 65% on day 30 for both conditions relative to the original activity on day 0).

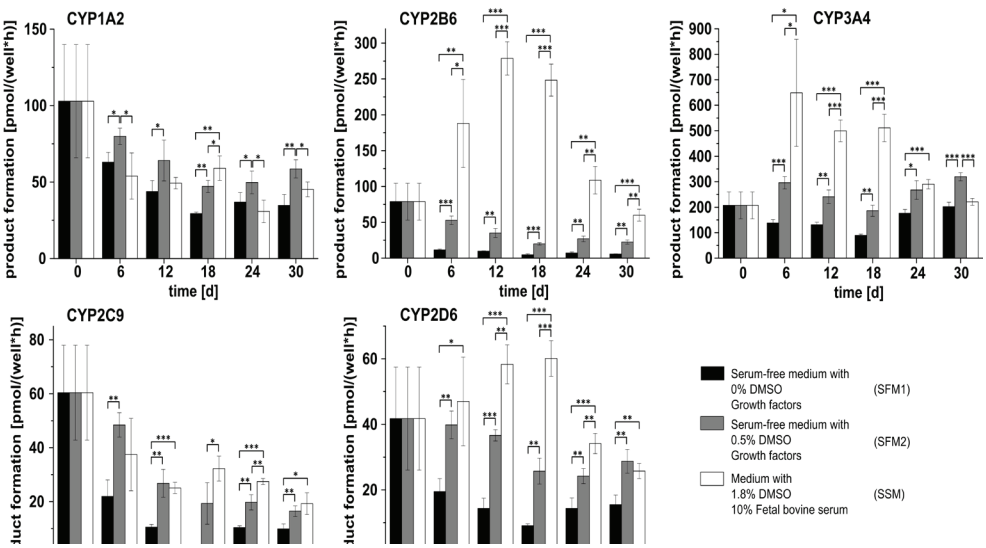


Figure 4.22 *Activities of CYP1A2, CYP2B6, CYP3A4, CYP2C9 and CYP2D6 enzymes in HepaRG cells during long-term cultivation with daily medium renewal. Error bars indicate standard deviation (n = 3). *, **, *** indicate p < 0.05, p < 0.01 and p < 0.001, respectively (Klein et al., 2013).*

Under these conditions, we analysed the extracellular metabolome (amino acids, glucose, pyruvate and lactate) of HepaRG cells. The uptake/production of various amino acids is depicted in *Figure 4.23*. The lactate/glucose ratios on days 6 and 30 were identical for SSM, SFM1 and SFM2.

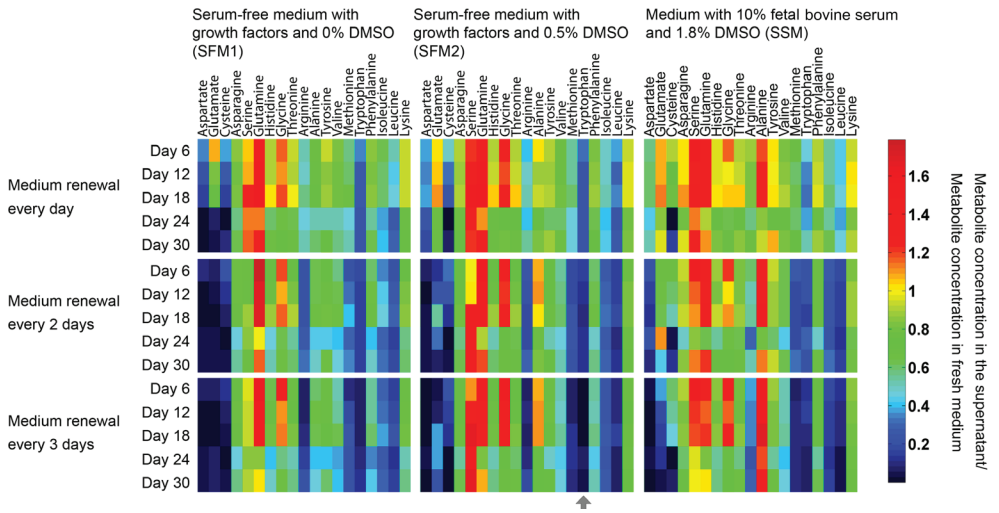


Figure 4.23 *A heat map showing the ratio of amino acid concentrations in the supernatants upon medium renewal every day, second or third day versus amino acid concentrations in the fresh medium, given for each investigated time point. An orange to red colour indicates production of amino acids, a yellow colour indicates that the amino acid was neither consumed nor produced. Green to blue colour indicates increasing consumption (Klein et al., 2013).*

Comparison of urea and albumin production after 30 days of cultivation shows that HepaRG cells exhibited high remaining activities for most conditions (*Table 4.10*). At day 30, urea production was between 61% and 366% (SFM1 and SSM, respectively) and albumin production was between 45% to 75% (SSM and SFM1) relative to respective productions on day 0.

Table 4.10 *Percentage remaining urea and albumin production after 30 days cultivation relative to day 0.*

Medium	Urea	Albumin
SFM1	61%	75%
SFM2	103%	65%
SSM	366%	45%

In addition to 2D HepaRG cultures, we further characterised HepaRG 3D spheroid cultures with respect to short-term and long-term cultivation. Morphological analysis was performed using light microscopy and transmission electron microscopy. In *Figure 4.24* electron microscopy

pictures of HepaRG spheroid cross-sections are depicted. Typical structures found *in vivo* were also found in 3D HepaRG spheroids. This includes typical liver structures such as bile canaliculi and microvilli (*Figure 4.24*A/B) as well as transport vesicles found inside the bile canaliculi, indicating that HepaRG cells have an intact transport within these channels.

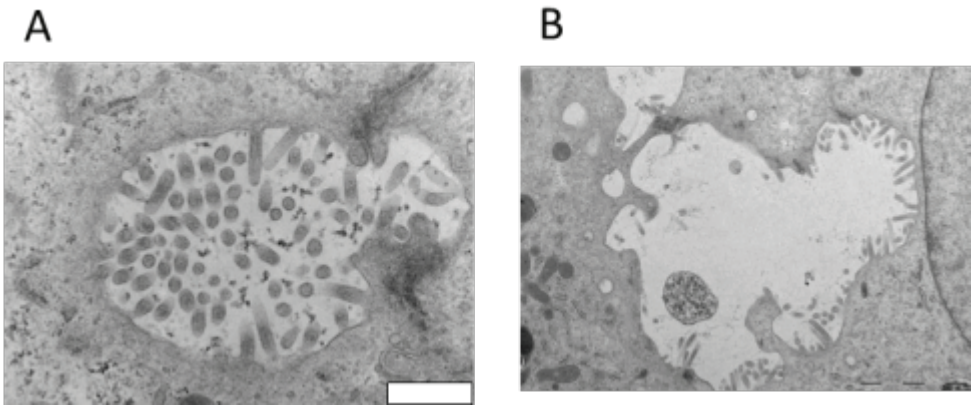


Figure 4.24 *Transmission Electron Microscopy of a bile canaliculus with microvilli in a HepaRG spheroid (2000 seeded cells, 200 µm diameter). White bars represent 500 nm (A), 2 µm (B).*

As with 2D cultures, we investigated the viability of 3D spheroids of HepaRG cells after 30 days of cultivation under serum-free (GF) and serum-supplemented (Serum) conditions. For both culture conditions, the viability was comparable to the viability at day 2. The cultures remained viable for at least 30 days (*Figure 4.25*). Thus, serum-free and serum-supplemented conditions are suitable for long-term cultivation of spheroids.

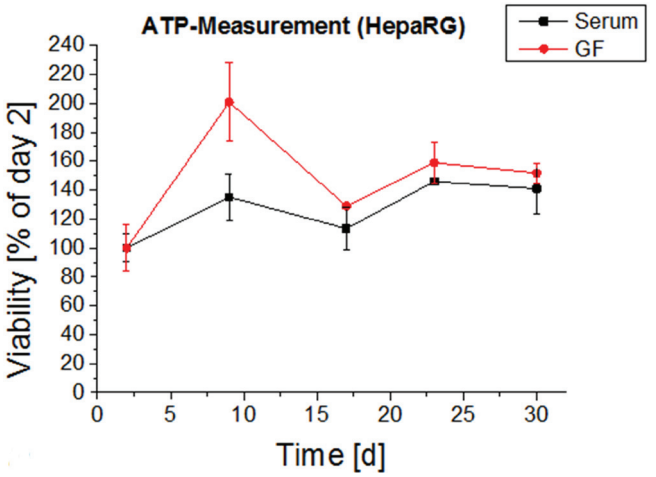


Figure 4.25 *Viability of HepaRG spheroid cultures (2000 seeded cells, 200 µm diameter) during 30 days cultivation upon maintenance in different media with medium renewal three times a week. GF = Growth factor supplemented medium, Serum = Medium supplemented with 10% fetal bovine serum (FBS). Error bars indicate standard deviations (n = 3). Viability is given in percentage relative to HepaRG culture viability on day 2.*

3D Organotypic Co-Cultures for Long-Term Toxicity Studies

As the liver does not only consist of hepatocytes, **NOTOX** investigated the possibility of using co-culture systems with HepaRG and non-parenchymal cells (NPCs) in spheroid format as an alternative long-term *in vitro* method. Differentiated human HepaRG cells from Biopredic (Rennes, France) and non-parenchymal cells from CelsisIVT (now BioreclamationIVT; Westbury, NY, US) were cultured as spheroids using the GravityPLUS™ system (InSphero AG, Switzerland) at different ratios (1:2 up to 1:16, non-parenchymal cells(NPC):HepaRG) for up to 2 weeks, whereby hepatocyte function and spheroid morphology were assessed. The total number of cells was 2000, as previously optimised. Spheroid morphology was maintained during the 14 day cultivation period (*Figure 4.26*); albumin production remained more stable in the 1:2 configuration (NPC:HepaRG) in comparison to cultures containing HepaRG cells alone (*Figure 4.26*).

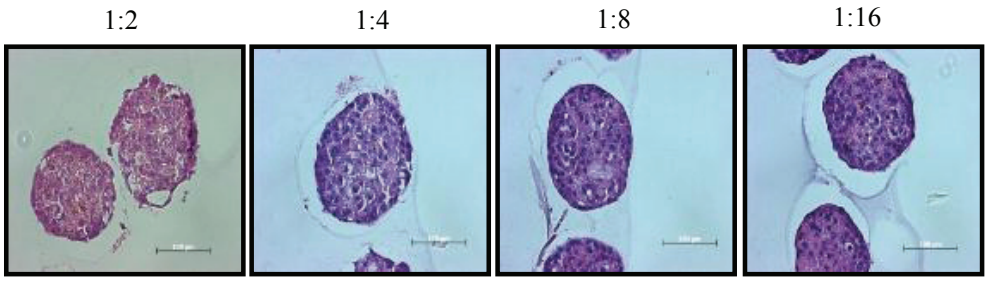


Figure 4.26 *H&E staining of non-parenchymal cells (NPCs)/HepaRG co-cultures after 14 days of cultivation. The NPC:HepaRG ratio is specified above each image.*

Importantly, identification of NPCs could be done at this NPC:HepaRG ratio by CD68 staining of liver macrophages (Kupffer cells) and MRP2 staining identifying the bile canalicular structures (as shown in *Figure 4.27*).

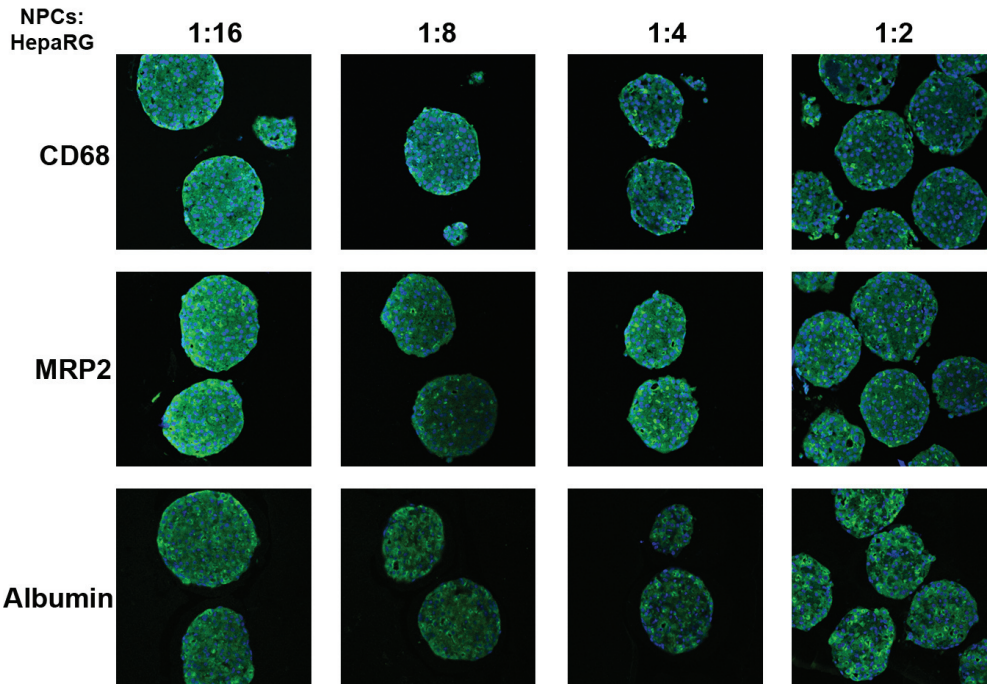


Figure 4.27 *14-day-old spheroid co-cultures of HepaRG and NPCs were stained with CD68, MRP2 and an albumin antibody for the identification of Kupffer cells, bile canaliculi and hepatocytes, respectively.*

We also investigated the liver specific functions of these co-cultures; the results are presented in *Figure 4.28*. It was demonstrated that the HepaRG cells in co-cultures maintain albumin and urea production for at least 10 days.

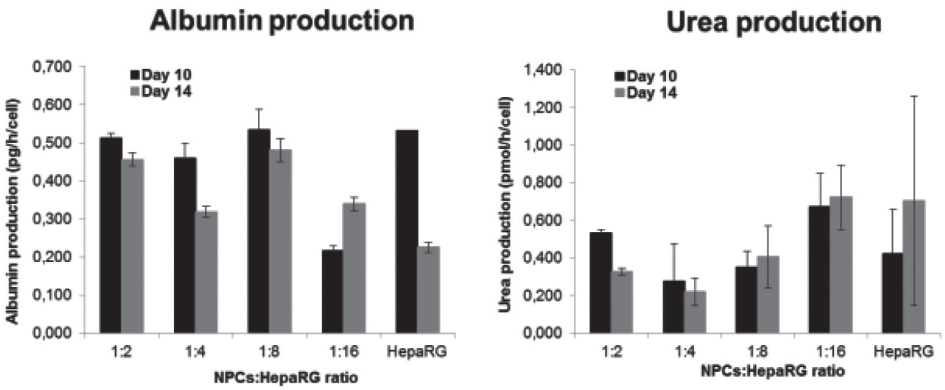


Figure 4.28 *Albumin and urea production in NPC:HepaRG spheroid co-cultures after 10 and 14 days in culture (n = 6 spheroid).*

4.7.4 Long-term Repeated Dose Toxicity Screening for Selected Compounds

An initial long-term repeated dose toxicity study on valproic acid in 2D HepaRG cultures using serum-free cultivation conditions was carried out. *Figure 4.29* presents the resulting dose response curves for different treatment times (24 h and two weeks). The 24 h EC_{50} values for valproic acid obtained by the concentration response curves were 21 (± 3.4) and 24 mM (± 3.6) upon pre-cultivation either in SSM or SFM2. After 2 weeks of repeated dose application (7 doses) in SFM2, the EC_{50} value decreased to 1.4 mM (± 0.2).

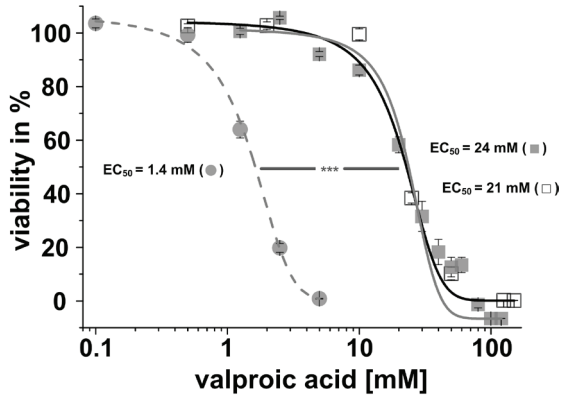


Figure 4.29 *Concentration response curves upon valproic acid treatment for 24h and 2 weeks (new dose every second day, 7 doses in total) under serum-free conditions. (□) shows dose response curve for HepaRG cells pre-cultivated under standard cultivation conditions. (■) and (●) show dose response curves on HepaRG cells for 24 h and 2 weeks respectively, with pre-cultivation of 4 days according to Figure 4.21. All toxicity assays were carried out in the absence of serum. Error bars indicate standard deviations (n = 3). *** indicates significance at p<0.001 (Klein et al., 2013).*

Following the 2D HepaRG experiments on valproic acid, the same dosing regimen was applied to 3D HepaRG spheroids. An initial experiment indicated that 3D spheroids are more sensitive to this compound compared to 2D cultures (*Figure 4.30A*), EC_{50} = ~0.5 mM compared to EC_{50} = ~1.4 mM (*Figure 4.29*), which was also confirmed in a second experiment (EC_{50} = ~0.7 mM; *Figure 4.30B*).

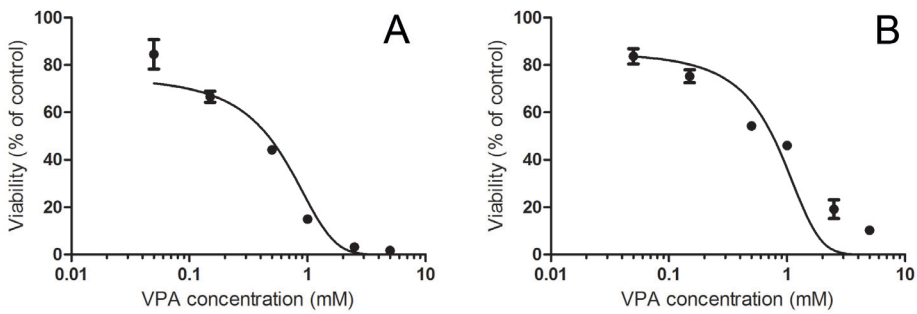


Figure 4.30 *HepaRG spheroids were exposed to a concentration range of valproic acid (VPA) for 14 days and viability was assessed by ATP measurement. Experiments represented in A and B were performed on different days and with different batches of HepaRG cells. Data are presented as mean +/- S.E.M., n = 4 spheroids.*

ATP measurements indicate decreased viability with increasing concentration of valproic acid, and also with time of exposure (*Figure 4.31A*). When corrected for viability, the levels of total glutathione are not greatly affected by valproic acid treatment with the exception of 0.5 mM valproic acid after 2 days of treatment (*Figure 4.31B*), which may suggest glutathione depletion prior to the onset of toxicity.

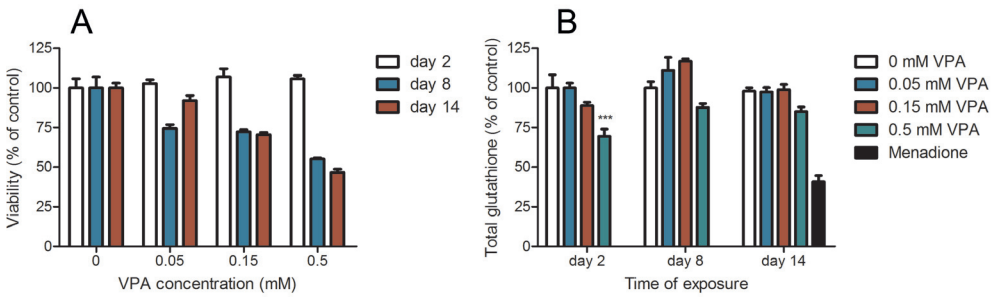


Figure 4.31 *HepaRG spheroids were exposed to a concentration range of VPA for 2, 8 and 14 days (dosing every 2nd day) prior to viability assessment by ATP measurement (A) and glutathione levels (B). All values are expressed as % of corresponding control at each time point and are represented by mean ± S.E.M. where n = 3-4 spheroids. Total glutathione values have also been corrected for viability; exposure to menadione (100 µM, 2 h) served as a positive control.*

In order to assess the effects of chlorpromazine exposure on HepaRG spheroids morphology and functionality, we exposed them to 100 µM chlorpromazine for 24 h. Treated and untreated spheroids were prepared for confocal microscopy and stained with rhodamine/phalloidin (F-actin) and DAPI (nuclei). In the case of untreated spheroids, we found significant accumulation of F-actin at the cell to cell contacts and inside the bile canaliculi. The exposure to 100 µM chlorpromazine induced cell death as detected by shrinking nuclei and collapse of the bile canaliculi (*Mueller et al., 2014*; *Figure 4.32*).

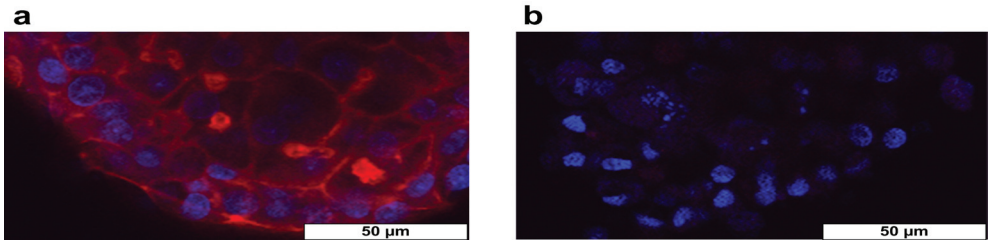


Figure 4.32 *Confocal microscopy on (a) untreated HepaRG spheroid, (b) HepaRG spheroid exposed to 100 µM chlorpromazine which corresponds to the EC_{50} value for 24 h. Rhodamine/phalloidin (red, actin) and DAPI (blue, nuclei) staining is shown. Scale bars represent 50 µm (Mueller et al., 2014).*

The co-culture of HepaRG cells and non-parenchymal cells allows for additional investigation of the effects of inflammatory stress combined with drug exposure. Trovafloxacin is a drug that induces liver injury, which has been linked to immune system activation. With this in mind, spheroid co-cultures of HepaRG cells and non-parenchymal cells were established and after 6 days of formation/stabilisation, spheroids were exposed to trovafloxacin with and without the addition of lipopolysaccharide to simulate a bacterial infection and induce an inflammatory response for 72h. Assessment of ATP levels following this exposure revealed a synergistic sensitisation of the spheroids exposed to both trovafloxacin and lipopolysaccharide compared to exposure to either lipopolysaccharide and trovafloxacin only (*Figure 4.33*).

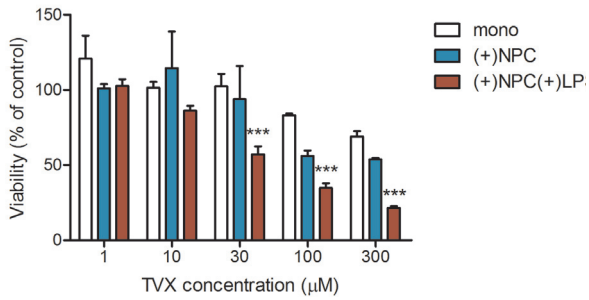


Figure 4.33 *HepaRG mono- and non-parenchymal cell (NPC) co-cultures were exposed to trovafloxacin (TVX) for 72h, with medium change every day, with and without the addition of lipopolysaccharide (LPS) at the first dose administration. Viability was determined by measuring ATP levels. Data represents mean ± S.E.M (n = 4 spheroids). Significance was determined by 2-way ANOVA where *** indicates p-value < 0.001.*

For comparative purposes, we established spheroid systems based on primary human hepatocytes in the presence or absence of non-parenchymal liver cells (*Figure 4.34*). The data show good preservation of the hepatocyte-based spheroids with additional effects on trovafloxacin toxicity following incubations with lipopolysaccharide.

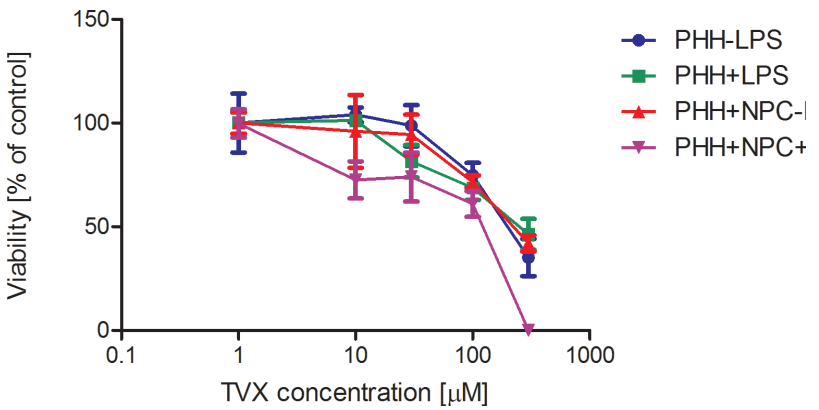


Figure 4.34 *Viability of primary human hepatocyte (PHH) spheroid cultures after exposure to trovafloxacin in the presence or absence of non-parenchymal cells (NPCs) or lipopolysaccharide (LPS) for 96 h. Data represents mean ± S.E.M (n = 4-5 spheroids).*

The optimised analytical strategies detailed above were applied to a long-term experiment on valproic acid effects at three different doses (**SEURAT-1** case study, see chapter 3.4.2). The cultivation was performed in a centralised manner, that was similar to the first **NOTOX** experiment (pilot study) aimed at the determination of acute toxic effects of acetaminophen (*Noor et al., 2013*). The experiment was carried out in serum-free conditions. Samples were taken for each of the different '-omics' measurements at day 0, 2, 8 and 14 and distributed among the participating collaborators. The analyses of samples and measurements are in progress.

4.7.5 Modelling

Metabolic Network Modelling

The effects of different cultivation conditions on the intracellular fluxes were determined using metabolic flux analysis. Several reaction and transportation rates are depicted in a flux distribution map (*Figure 4.35*). Reaction rates in the glycolytic pathway of HepaRG cells were significantly lower in serum-supplemented media (SSM) as compared to serum-free media (SFM). Accordingly, lactate secretion was lower for cells maintained in SSM. Generally, glycolytic activity increased over time for all conditions. For cells cultivated in media without DMSO (SFM1), approximately 40% of the glycolytic pyruvate was converted to lactate; for cells kept in 0.5% and 1.8% DMSO respectively (SFM2 and SSM), about 50% to 55% of glycolytic pyruvate was metabolized to lactate.

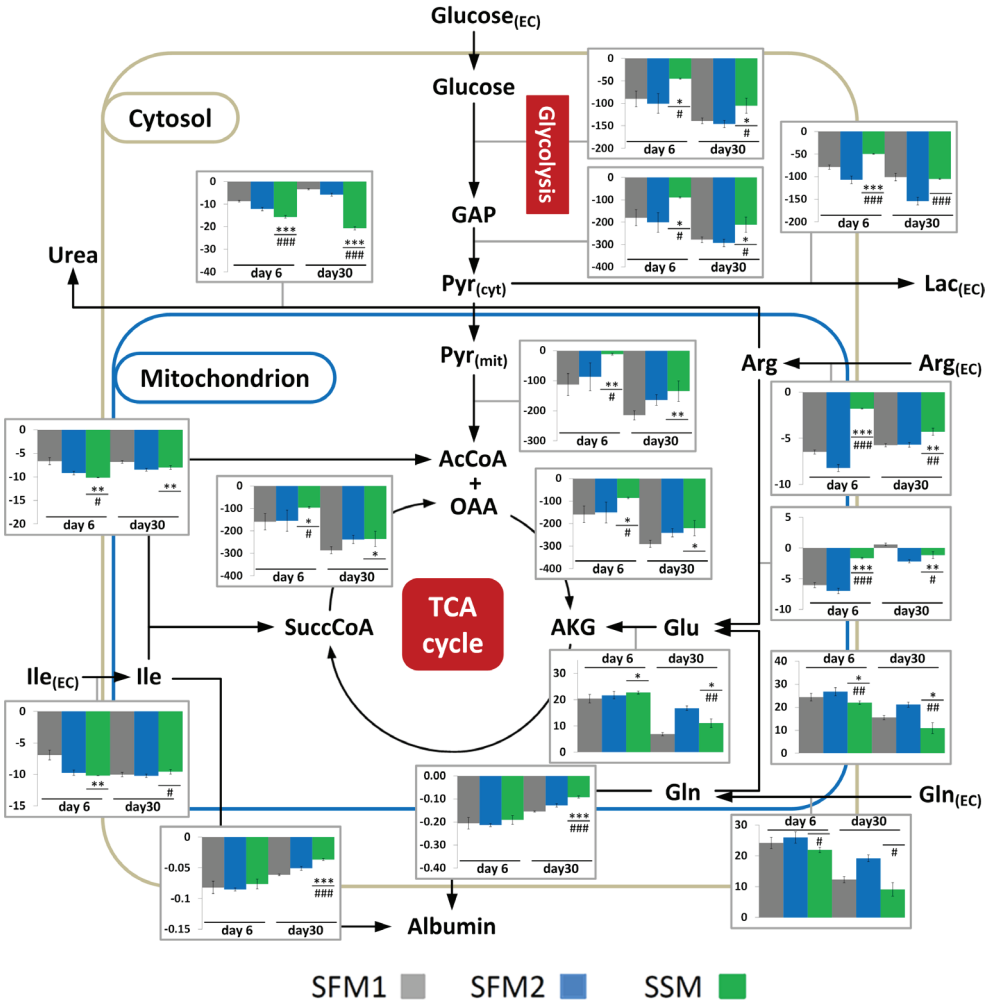


Figure 4.35 *A flux distribution map of HepaRG cells upon long-term cultivation for days 6 and 30 for SFM1 / 2 (serum-free media with growth factors and 0% or 0.5% DMSO, respectively) and SSM (serum-supplemented medium with 1.8% DMSO). Negative values indicate fluxes in the direction of the arrow and positive values in the opposite direction. Error bars indicate standard deviation (n = 3). *, **, *** (comparison of SSM to SFM1) / #, ##, ### (comparison of SSM to SFM2) indicate significance at p<0.05, p<0.01 and p<0.001, respectively. GAP, glyceraldehyde 3-phosphate; Pyr, pyruvate; AcCoA, acetyl coenzyme A; OAA, oxaloacetate; AKG, α-ketoglutarate; SuccCoA, succinyl coenzyme A; Lac, lactate; Glu, glutamate; Gln, glutamine; Arg, arginine; Ile, isoleucine; cyt, cytosolic; mit, mitochondrial; EC, extracellular; TCA, tricarboxylic acid.*

All flux estimations involving ^{13}C metabolic flux analysis were performed using the metabolic network shown in *Figure 4.36*. Reactions of the glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA cycle), anaplerosis, amino acid degradation and synthesis, glycogen degradation and lipid metabolism, reactions of the urea cycle were included.

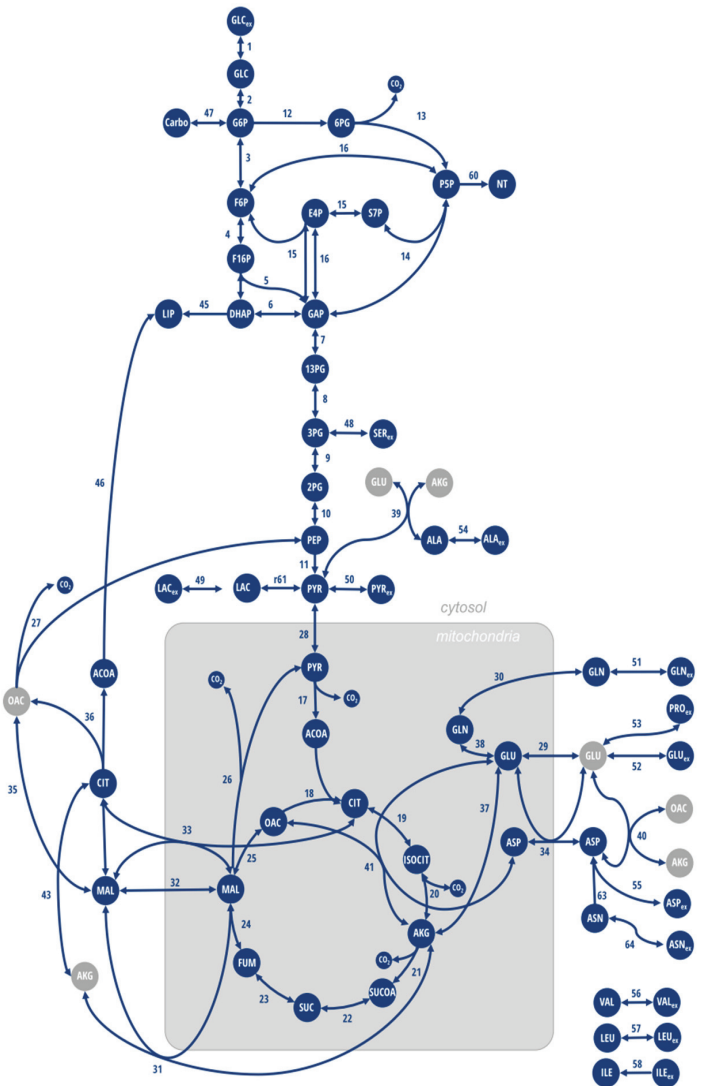


Figure 4.36 *Metabolic network model used for the estimation of fluxes with ^{13}C metabolic flux analysis. Abbreviations: GLC, glucose; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F16P, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; 13PG, 1,3-bisphospho-glycerate; 3PG, 3-phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; PYR, pyruvate; 6PG, 6-phospho-*

gluconate; P5P, pentose 5-phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; ACOA, acetyl-CoA; CIT, citrate; ISOCIT, isocitrate; AKG, alpha-ketoglutarate; SUCOA, succinyl-CoA; SUC, succinate; FUM, fumarate; MAL, malate; OAC, oxaloacetate; ASP, aspartic acid; GLU, glutamic acid; GLN, glutamine; PRO, proline; ASN, asparagine; VAL, valine; LEU, leucine; ILE, isoleucine; SER, serine; ALA, alanine; LAC, lactate; NT, nucleotide; ex, extracellular.

Biokinetics and In Vitro to In Vivo Extrapolation (IVIVE)

Biokinetics analysis demonstrated that unspecific binding mechanisms, which depend on drug's physicochemical properties and physiological charge, could significantly contribute to the *in vitro* dynamic drug distribution. This should be taken into consideration in the experimental design (*Figure 4.37*).

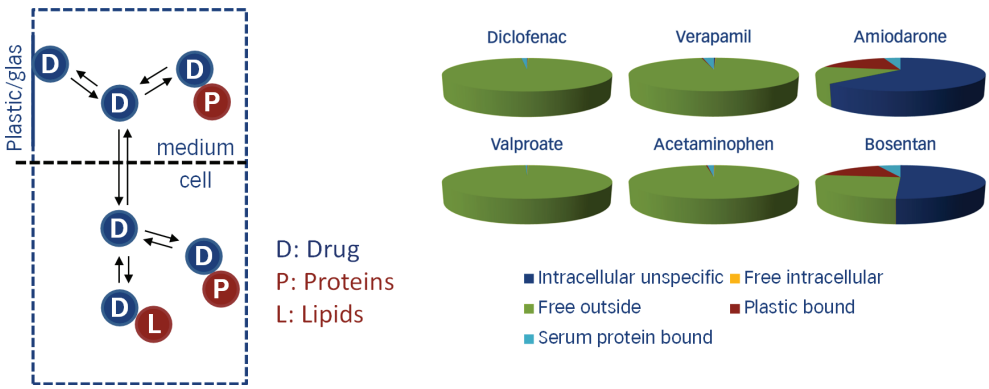


Figure 4.37 *Determination of drug disposition in vitro. Depending on the compound's physico-chemical properties (logP of micro-species with corresponding charge at physiological pH), the substance binds non-specifically to cellular and extracellular macromolecules (lipids, proteins) and to the plastic or glass surface (assumptions: low drug concentrations with no binding saturation).*

In vitro studies for toxicity assessment only provide limited information about potential risks of compounds *in vivo* as certain parameters, like bioavailability, are not adequately reflected in *in vitro* experiments (*Rotroff et al., 2010*). To overcome the gap between *in vitro* and *in vivo* experiments, **NOTOX** partners started a collaboration with Silvia Maggioni (ToxBank, Mario Negri Institute, Italy). This collaboration aimed to combine Maggioni's quantification of drug metabolites in culture supernatants with IVIVE, similar to previously reported studies using *in vitro* data on 2D HepaRG cultures (*Rotroff et al., 2010; Wetmore et al., 2012)*. As a result, the oral equivalent dose (OED) was obtained, i.e. the dose which results in *in vivo* concentrations

corresponding to the *in vitro* effective concentration (EC) of interest (*Rotroff et al., 2010*), for acute and long-term treatment. Further, a virtual population that considered differences in physiological characteristics was established and applied in the analysis.

OEDs based on uptake rates and time-dependent EC_{10} profiles for acute drug exposure and for 28 days of drug treatment are given for valproic acid and bosentan (*Figure 4.38*), respectively. The results show that valproic acid does not elicit acute toxicity in patients when the recommended daily dose is applied (*Figure 4.38A*). In the case of long-term treatment, the OED based on ATP assay is slightly lower than the recommended daily dose (*Figure 4.38B*), indicating that intake of the drug could potentially result in hepatotoxicity within 28 days of treatment in a certain percentage of the population. For bosentan, the OED for long-term treatment is, similar to valproic acid, lower than for acute toxicity (*Figure 4.38C*). Further, the OED of bosentan was found to be below the recommended daily dose for the long-term treatment (*Figure 4.38D*). This indicates that bosentan-induced hepatotoxicity could affect a higher percentage of the population compared to valproic acid. In general, the agreement between IVIVE using HepaRG *in vitro* data and human data looks excellent.

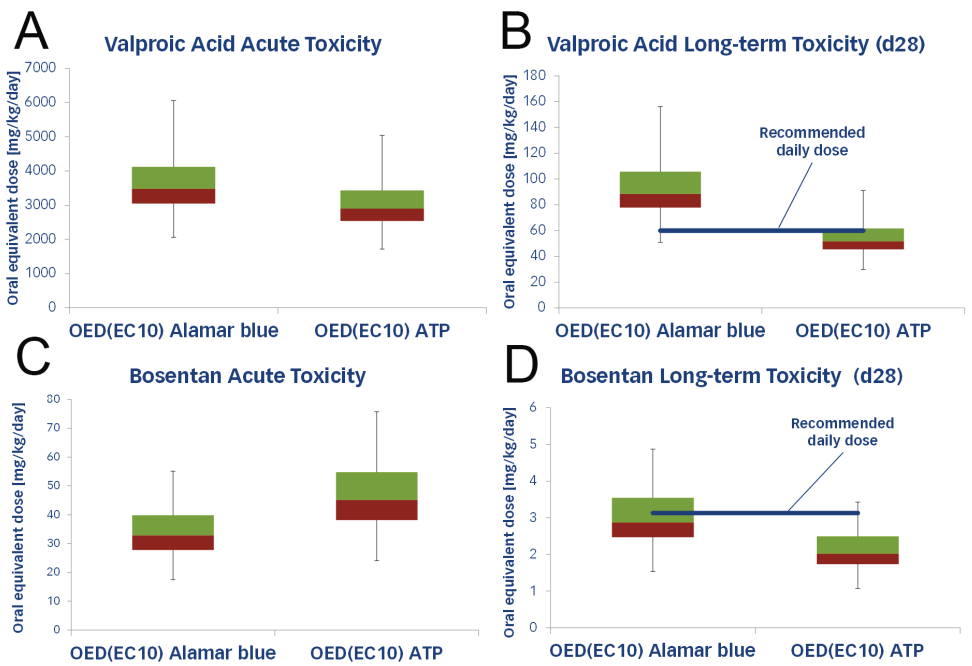


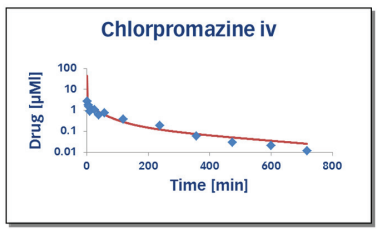
Figure 4.38 *Application of the oral equivalent dose (OED) approach for the estimation of safe/critical doses of valproic acid (upper panels) and bosentan (lower panels). OEDs were estimated according to Wetmore et al. (2012), by applying effective concentrations resulting in 10% of the assay response (EC_{10}) from in vitro dose response data measured in acute*

(left panels) and long-term toxicity (right panels) experiments based on two different cellular viability assays (alamarBlue, ATP) in 2D HepaRG cultures. A virtual population was applied and safe doses were estimated for each individual. Inter-individual variability can be seen in the box-and-whisker plots in each figure panel.

Physiologically Based Pharmacokinetic (PBPK) Modelling

A **NOTOX** partner uses in-house validated whole body physiologically-based pharmacokinetic (PBPK) models which allow the dynamic estimation of safe doses from dynamic drug profiles in all organs of interest. Next to the OED method, the PBPK model is used for the estimation of safe doses using drug degradation rates and physicochemical properties of the compound as input dose response data. The model simulations show which doses have to be administered through different routes in order to reach effective concentrations in the tissues of interest equivalent to the effective doses determined *in vitro* (*Figure 4.39*).

PK Prediction [Dose 4 mg/kg]



Tissue	Critical Dose [mg]
Brain	53.33
Gut	47.58
Heart	140.30
Kidney	118.48
Liver	39.70
Lung	93.64
Marrow	102.12
Muscle	240.91
Pancreas	126.97
Spleen	179.09
Stomach	72.12

PK Profiles [Dose 4 mg/kg]

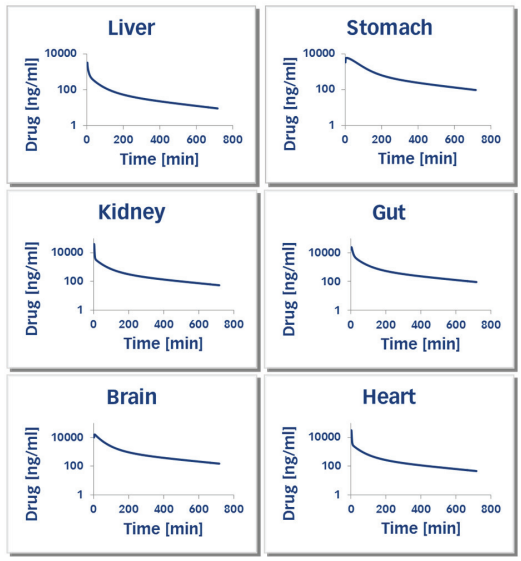


Figure 4.39 *Prediction of tissue-specific critical intravenous (IV) drug doses in rats. The validated rat PBPK model, here applied to the simulation of intravenous chlorpromazine administration (top left), was used for the determination of organ concentration profiles (right side) and critical doses. In this case, tissue-specific effective concentrations are compared with* EC_{50} *of in vitro experiments and potentially critical (or equivalent) doses are derived (bottom left).*

The respective dose, which corresponds e.g. to the 'no observed adverse effect level' (NOAEL), can be assumed to be safe; doses exceeding this point should be considered critical. Furthermore, this approach allows the consideration of dose response data for different markers, e.g. lipid accumulation for steatosis or NADH-depletion for cytotoxicity. This approach is suitable for analysis of single as well as repeated dosing in long-term toxicity studies.

The long-term-toxicity model of valproic acid is currently under construction for the liver compartment of the PBPK-model described above. This would allow for verification against the experimental data and also validation against *in vivo* data.

Spatio-Temporal Modelling

Spatio-temporal models are currently under development by a **NOTOX** partner, focusing on monolayer, sandwich and spheroid cultures and implementing data from pilot experiments. The cellular acetaminophen model, which is adapted on the HepaRG pilot experiment data, was prepared for integration into organoid models by defining the links in the model for the communication with the surrounding culture and organoid tissue models. The model is currently being tested. We developed an Individual Based Cell (IBC) model that can mimic the formation of tissue for monolayers, sandwich cultures and spheroids. The model incorporates cell growth, cell adhesion, ECM production and migration. The model qualitatively reproduced the observed process of tissue formation, starting from the cell seeding to the formation of spheroids. Positive correlation between asphericity and cell number could also be reproduced. We also developed a Deformable Cell Model (DCM) that can adapt its shape; this behaviour has been observed experimentally when cells are confined by ECM or other cells. This model can quantitatively mimic the intracellular spaces between cells, as well as the local mechanical stress that individual cells experience.

Integrating compartment models in CellSys II allows for toxicity testing simulations in agent-based models. Using the PDE solver for diffusion coupled to our individual based cell model and the intracellular module, we found that there is little or no acetaminophen concentration gradient in the spheroid. This is different from oxygen, for which a concentration gradient is established leading to oxygen limitation for spheroids of diameters larger than 200 μm. Using the calibrated parameters from the cellular model we obtained viability results that agree with the monolayer experiments performed by other **NOTOX** partners (*Figure 4.40*, right). In the model simulations, behaviour of individual cells differs according to variability.

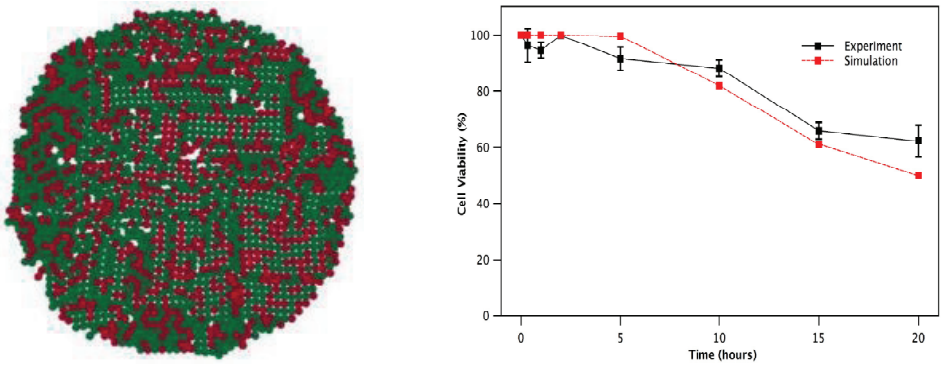


Figure 4.40 *Prediction of live-dead staining in 2D culture. Comparison of cell viability as a function of time in a (monolayer) 2D HepaRG culture (left side: model snapshot; red: dead cells, green: viable cells) between model simulation and experiment (right side) after exposure to 15 mM acetaminophen.*

NOTOX is also analysing SEM/FIB imaging. *Figure 4.41* shows one slice from such a dataset. During processing, we segment the datasets to highlight essential elements and then reconstruct a 3D spatial model from the individual slices of the relevant parts of the spheroid. Such a 3D model will be subsequently analysed by other consortium partners with respect to the extracellular features that play a role in spheroid formation as well as modifications of the intracellular ultrastructure induced by drug exposure.

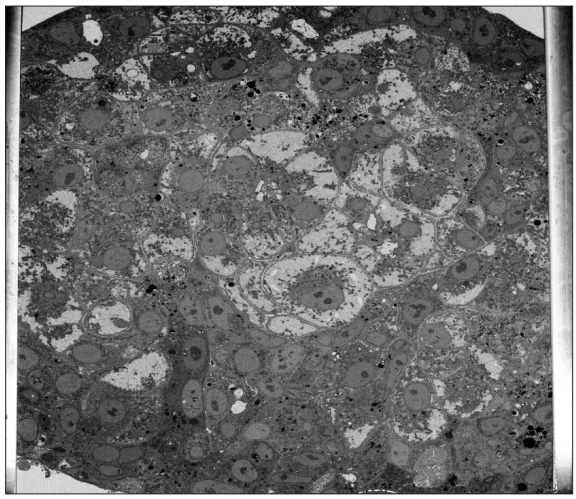


Figure 4.41 *A slice from a FIB/SEM dataset which is currently being processed. The goal of the processing is to reconstruct a spatial 3D model that will highlight the key aspects of the overall spheroid structure as well as influence of the drug exposure on intracellular ultrastructure.*

4.7.6 Cross-cluster Cooperation

Various cross-cluster collaboration possibilities were identified and discussed during the **SEURAT-1** Annual Meeting in February 2014 (Barcelona, Spain). Some cross-cluster joint case studies may be possible. Collaboration with *HeMiBio* using HepaRG co-cultures with human stellate cells has been discussed. Discussions are ongoing for applying methods developed and tested in **NOTOX** to cells available from *SCR&Tox*. Further collaborations with ToxBank and COSMOS will continue and be strengthened in the coming year.

Besides these recently planned activities, the **NOTOX** consortium emphasised the importance of **SEURAT-1** cross-cluster cooperation on multiple occasions during the last year. Various collaborative efforts were initiated and could be intensified between **NOTOX** and the other **SEURAT-1** cluster projects. Three **NOTOX** partners are also participating in other cluster projects, namely: The Leibniz Research Centre for Working Environment and Human Factors (in DETECTIVE), the Karolinska Institutet (in *SCR&Tox*), and Insilico Biotechnology (in COSMOS). These partners are thus interacting with other cluster projects intensively and on a routine basis. For example, Insilico Biotechnology cooperates closely with the COSMOS project both in-house as well as with other research groups (including the European Commission's Joint Research Centre (Ispra, Italy); Institut National de l'Environnement Industriel et des Risques, France). They are focusing on the combination of cellular network models with structured organ models and PBPK models for the simulation of drug distribution in the whole body.

Furthermore, the **NOTOX** consortium contributed actively to **SEURAT-1** case studies, namely the case study *Evaluation of valproic acid (VPA) induced steatosis (MoA) in HepaRG cells*. The **NOTOX** project, in cooperation with the ToxBank consortium, also organised several on-site workshops to foster data integration and improve data management, exchange and implementation in models. Moreover, ToxBank coordinator Barry Hardy (Douglas Connect) attended the 5th progress meeting of the **NOTOX** consortium. **NOTOX** partners contributed also to the second *HeMiBio* joint meeting on 'Bioreactors and Cell Engineering' in Gent in September 2013, with delegates from *SCR&Tox*, **NOTOX** and DETECTIVE.

The **NOTOX** consortium has been active at joint **SEURAT-1** events and contributed the five following posters to the poster session at the **SEURAT-1** Annual Meeting in Lisbon in March 2013:

- Proteomics for detection of drug-induced toxicity using HepaRG cells;
- Toxicoepigenomics: Transcriptional and epigenetic profiles of primary liver cells and *in vitro* model;
- Systems toxicology approach to assess acute effects of acetaminophen on HepaRG *in vitro* cultures;
- 3D organotypic cultures of human HepaRG cells: a tool for *in vitro* toxicity studies;

➠ Multi-scale modelling for individualised spatiotemporal prediction of drug effects.

The **NOTOX** coordinator (Elmar Heinzle) represented the consortium at the SEP Meetings in Lisbon in March 2013, and in Ispra in June 2013. During these meetings he was supported by Fozia Noor (Lisbon) and Jens Niklas (Ispra) from the **NOTOX** group. Elmar Heinzle and Jens Niklas also participated in the 'SEURAT-1 meets Tox21' workshop in Ispra (see section 5.3.1). Finally, **NOTOX** contributed the following two posters to the 1st **SEURAT-1** Stakeholder Event in Brussels on September 5, 2013, at which Heinzle gave an oral presentation *System modelling for human toxicity prediction*:

➠ *In silico* solutions for predictive toxicity - multi-scale modelling and *in vitro* to *in vivo* extrapolation;

➠ Modelling spheroid formation for later organotypic toxicity prediction.

4.7.7 Expected Progress within the Fourth Year

Multi-omics experiments: Integrated data analysis of the multi-omics experiment on valproic acid will be carried out. Further experiments with other selected compounds will be planned and executed. Complementary experiments to add to the results of the previous experiments will be carried out to obtain better multi-scale mechanistic information.

3D organotypic cultures for long-term repeated dose toxicity assessment: The 3D spheroid cultures will be further developed, characterised and tested, especially for long-term repeated dose toxicity studies. A range of cell compositions will be evaluated and different endpoints will be used depending on the study compound. HepaRG spheroids will be compared to similar spheroids made using primary human hepatocytes and non-parenchymal cells. Intracellular signal transduction systems activated by the compounds will be identified and metabonomics evaluations will be carried out.

A special emphasis will be on advanced methods of IVIVE, applying *in vitro* testing using human HepaRG spheroids together with spheroid-*in vivo* extrapolation models of mouse. Detailed characterisation of primary mouse hepatocyte spheroids will be carried out and compared with existing *in vivo* mouse data as well as with HepaRG spheroids.

Modelling: The modelling efforts will be intensified as results from experiments are now available. In the first step, valproic acid metabolism and adverse effects caused will be modelled, verified against experimental time-series data on HepaRG cells and validated against pharmacokinetics *in vivo* data.

Furthermore, the spatio-temporal modelling of spheroid formation in the hanging drop setting will be continued to gain insights into the spheroid architecture.

After experimental validation of *in vitro* predictions of acetaminophen toxicity, the model will be extended from the *in vitro* monolayers and spheroids to *in vivo* liver lobules. The intracellular acetaminophen toxicity model will be applied to each cell of the agent-based spatio-temporal model of a liver lobule, following the same approach as for *in vitro* predictions. Cell-to-cell variability and blood flow will be taken into account.

4.7.8 Future Perspectives

We see a bright future for systems-oriented methods in toxicology. A broad '-omics'-based analysis will 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 the further development of 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.

It is projected that the **NOTOX** project will eventually develop easily applicable methods of analysis that can be readily transferred to other cellular systems, such as 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 (even functional organoids), are presently limited in their applicability due to the lack of sufficiently sensitive analytical techniques. These cultures 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 predictive power. In terms of systems biology, 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. The ultimate 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, in order to recognise 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, Prizes and other Achievements

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

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

The **NOTOX** publication *Klein et al., 2013* (see above) has been selected as a highlight in the Journal of Chemical Research in Toxicology, in the special issue on Systems Toxicology, March 2014 (Dahlmann, H.A. (2014): Spotlight. *Chem. Res. Toxicol.*, 26: 312-313).

Lukas Marselek, a former postdoc of **NOTOX** at the German Research Centre for Artificial Intelligence, has established an SME (Eyeon) in Prague in 2014.

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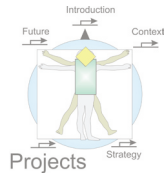
Introduction
Future
Context
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4.8 ToxBank: Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology

Emilio Benfenati, Roland Grafström, Barry Hardy, Pekka Kohonen, Glenn Myatt, Micha Rautenburg

4.8.1 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 for 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;

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

➠ 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') that have high-quality data and provide 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 internal compliance with procedures, 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, that characterise relevant physico-chemical 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, 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 data standards, 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 that support and service

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.

4.8.2 Main Achievements and Challenges in the Third Year

The **ToxBank** consortium has made considerable progress towards the Data Warehouse objectives. Specifically, based on extensive data gathering and analysis from all **SEURAT-1** consortia, a production version of the **ToxBank** Data Warehouse that provides access to all experimental, processed data and protocols alongside relevant public information has been implemented. This includes the development and/or customisation of web-based interfaces for linking and uploading data, including raw data, processed data and model results. All steps of any experiments are linked to protocols describing the procedures. A web-based user interface 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 to any intellectual property restrictions on access. The system has been implemented as a series of Representational state transfer (REST)-based web services, which enable interoperability with other systems across the cluster as well as with external resources.

Protocol guidelines have been developed and uploaded to the **ToxBank** Data Warehouse, providing definition, information on content, and guidance for the compilation, uploading and sharing of research protocols and standard operation procedures within the projects of the **SEURAT-1** Research Initiative. To support an integrated view of the data derived, processed or otherwise generated from experiments across the **SEURAT-1** Research Initiative as well as outside the cluster, **ToxBank** uses preconfigured templates for assay metadata (ISA-tab) and has proposed a standard file format for processed data. With this proposed standard, uploaded data can be used in **ToxBank** to support precise searching, as well as a consistent integrated analysis of the data over the entire cluster.

ToxBank has been supporting the preparation and upload of protocols and data into the Data Warehouse. So far, 23 protocols and five investigations have been uploaded into the Data Warehouse: Nine additional investigations have been prepared and are being reviewed with eight investigations currently being worked on. In addition to applying the ISA-tab approach to representing data, we are also requesting that each step of the experiment, including the processing of data, be precisely documented as either a research protocol or a standard operating procedure that should be uploaded separately into **ToxBank** and linked to the data. We are also requesting that any processed data should be formatted using the standardised fields discussed earlier. There are many benefits to using this approach. It ensures that all investigations are precisely documented to allow others to understand, repeat if necessary, as well as have confidence in the results. It will be impossible to perform any integrated analysis or safety assessment without the use of standardised data formats.

Selection criteria and standard operating procedures for data quality control, acceptance, processing and analyses of **ToxBank** Gold Compounds were published in a wiki (*ToxBank, 2014*). This collaborative reference compound database is based on the Semantic MediaWiki platform, and has been populated with information on a set of 51 compounds as of the end of 2013. Information and data on the compounds, including information about chemical identities, adverse effects, toxicity mechanisms and therapeutic targets, was incorporated into the wiki. These compounds are available as reference compounds for the **SEURAT-1** Research Initiative. In order to facilitate an integrated analysis of available data and evidence associated with reference compounds, this information needed to be collected, organised and systematically made available to the **ToxBank** Data Warehouse. Furthermore, analysis methods and tools were required in order to pursue goals of integrated data analysis, visualisation and interpretation. Finally, extensions to reference compounds and data into another chemical space (cosmetics) required developments, such as use cases achieving interoperability between the **ToxBank** and COSMOS databases.

Data on the **SEURAT-1** Gold Compounds were obtained from the literature and organised and made available through the ToxBank wiki and Data Warehouse. The data integration included transcriptomics data from TG-Gates, assay data from PubChem, and toxicokinetics data and parameters from the literature. Interoperability between **ToxBank** and OpenTox tools for analysis and the COSMOS database for *in vivo* data was advanced. Analysis methods for read-across, enriched meta-analysis of multiple '-omics' and functional data, background knowledge from GO ontologies and Kegg pathways, and pathway visualisation were developed and applied to the **SEURAT-1** Gold Compounds. This approach is reported in detail as the selected highlight of the year (see the following section 4.8.3).

Analytical methods based on LC-MS/MS were developed for doxorubicin, tamoxifen, amiodarone, bosentan, chlorpromazine, valproic acid and its metabolites. The methods were applied to the measurements of the Gold Compounds concentration in acute and long-term toxicity studies on HepaRG cells. The results are crucial for the evaluation of the actual concentration of the compounds at different time points during the experiments and to evaluate their availability to the exposed cells. These are required data for the calculation of the *in vitro* biokinetics.

4.8.3 Selected Highlight: Integrated '-omics' Analysis of SEURAT-1 Gold Compounds

Introduction

The Open TG-GATEs (Toxicogenomics Project-Genomics Assisted Toxicity Evaluation system) database from the Toxicogenomics Project (TGP) / Toxicogenomics Informatics Project is a public-private partnership initiated in 2002 by the Japanese National Institute of

Biomedical Innovation (NIBI), the Japanese National Institute of Health Sciences (NIHS), and fifteen pharmaceutical companies (*Uehara et al., 2010*). Chemicals were administered to rats or exposed to rat and human primary cultured hepatocytes, and the gene expression profiles in the liver and kidney of the animal or in the cultured cells were comprehensively analysed by microarray. In addition, data acquired to test biomarkers and analyse their mechanisms are included in TG-GATEs. Analysis of the high-quality large-scale toxicogenomics database has resulted in the development of more than 30 safety biomarkers (*Uehara et al., 2010*). Open TG-GATEs is a toxicogenomics database open to the public for researchers to utilise the research results, and releases the data of 170 compounds stored in TG-GATEs (*NIBI, 2014*). In Open TG-GATEs, it is possible to search toxicogenomics data by compound name or pathological finding. It is also possible to download gene expression data associated with phenotype data, such as pathological findings. This database was used to study gene expression changes induced by **SEURAT-1** Gold Compounds.

Read-across procedures, and the identification of adverse outcome pathways, are important use cases within the **SEURAT-1** Research Initiative. In order to support these cases, to reduce the workload of toxicological experts and to reduce the ambiguity of read-across procedures, we have developed and implemented a novel tool (http://aop.in-silico.ch) utilising information from the PubChem database (*PubChem, 2014*). PubChem is presently the most comprehensive database of chemical molecules and their activities against biological assays, and integrates almost all databases relevant in this area (e.g. CHEMBL, TG-GATES, ArrayExpress). At present it contains data for more than 40 million unique structures and more than 500,000 bioassays. It is therefore ideally suited as a central entry point for queries about small molecules and their impact on biological systems. Furthermore, PubChem is currently planned as a primary public deposition mechanism for data from the US National Toxicology Program, Tox21 (*Judson et al., 2014; Kohonen et al., 2014*).

Our overall goal was to develop a strategy for performing a meta- and pathway analysis of the **SEURAT-1** Gold Compounds using publicly available databases as a starting point. The approach is described in the following section and was applied to analyse multi'-omics' data generated by NOTOX (effects of acetaminophen on human liver cells) and functional data generated by DETECTIVE (effects of doxorubicin on cardiomyocytes as well as ochratoxin A and potassium bromate on kidney cells). However, as these data sets still remain confidential at the consortium level, the results of these analyses cannot be reported here. Instead, we have described our approach as a case study, using doxorubicin as a selected example.

Searching for Similar Compounds and Mechanisms

ToxBank partner In Silico Toxicology GmbH developed a novel tool that utilises information from the PubChem database to improve read-across studies using data of structurally similar compounds. The tool is available online (http://aop.in-silico.ch) and is at present able to:

- Search for all available biological data for a given chemical structure;
- Differentiate between assays with and without defined (gene or protein) targets (e.g. gene expression changes vs. acute toxicity);
- Identify targets affected by the query compound;
- Find structurally similar compounds (neighbours) within the PubChem database;
- Retrieve biological data for these neighbours;
- Distinguish between gene/protein targets and assay outcomes for the neighbours;
- Use neighbour data in order to predict targets likely to be affected by the query compound;
- Use neighbour data in order to predict assay outcomes of the query compound.

With the help of this tool, a toxicological risk assessor can identify possible adverse effects even in the absence of experimental data for the query compound. By using similar compounds as an additional source of information, the lists of affected/unaffected targets and individual assay outcomes are enriched significantly, which leads to improved information about possible mechanisms.

As large parts of PubChem data are not curated (human curation would be impossible for a database of this size) it is crucial to provide the means for a critical examination of search and prediction results. For this reason the user interface presents data in an intuitive tabular format with a clear distinction between (i) target and assay outcomes; (ii) measured data and predictions; and (iii) data from similar compounds.

Users are encouraged to use their expertise when interpreting query and prediction results and discard information that is likely to be wrong and/or irrelevant.

For read-across extrapolation we use a simplified version of the lazar algorithm (*Maunz et al., 2013*), which resembles traditional read-across procedures very closely. Automating read-across has the advantages of:

- Reducing the workload of toxicological experts by performing tedious and time consuming tasks (e.g. database searches) automatically;
- Reducing the ambiguity of read-across procedures by following well-defined algorithms;
- Extending the coverage of searches by performing cross-database searches utilizing all data aggregated in PubChem;

- Easy access to target/pathway information;
- Increased reproducibility of read-across predictions, by reducing *ad hoc* decisions and providing better data coverage.

Technically, the system provides: an (almost) OpenTox-compatible wrapper for the PubChem database which hides unintuitive internal PubChem data structures; a sophisticated caching system to speed up queries; and some hacks to enable fast large-scale similarity calculations.

Further development will depend primarily on feedback received from users. Possible directions include an improved depiction of gene/protein targets (graphical display of affected pathways), recalculation of results after expert corrections and reporting facilities.

The formal validation of target/assay predictions is still lacking and is foreseen in the future. Preliminary results indicate very high accuracies (>95%) for negative predictions (inactive assays, non-targets) and good accuracies (~80%) for positive predictions (active assays, targets). The difference between active and inactive accuracies originates from the composition of PubChem that contains predominately negative results. This makes it much easier to predict negative outcomes, but the preliminary positive prediction indicates that the system is indeed working as expected.

A proof-of-concept OpenTox-compliant algorithm for identifying relevant pathways through the OpenPhacts API was implemented. Among all **SEURAT-1** Gold Compounds, only two (aflatoxin B1 and chlorpromazine) participate in pathways, documented in OpenPhacts (which integrates WikiPathways content). Data analysis procedures could be further developed to take into account pathways information in order to estimate biological similarity and identify compounds with similar mechanisms.

Prototypic '-omics'-based Compound Assessment Workflow

Described below is an integrated data analysis workflow for toxicogenomics-based assessment of a compound (*Figure 4.42*). The '-omics'-based assessment can be combined with high-throughput screening (HTS) results e.g., from ToxCast or Tox21 projects retrieved via PubChem as outlined above or with structure-activity relationships (SARs) implemented in the OpenTox framework to generate a more complete assessment (*Hardy et al., 2010; Judson et al., 2014; Kohonen et al., 2014*).

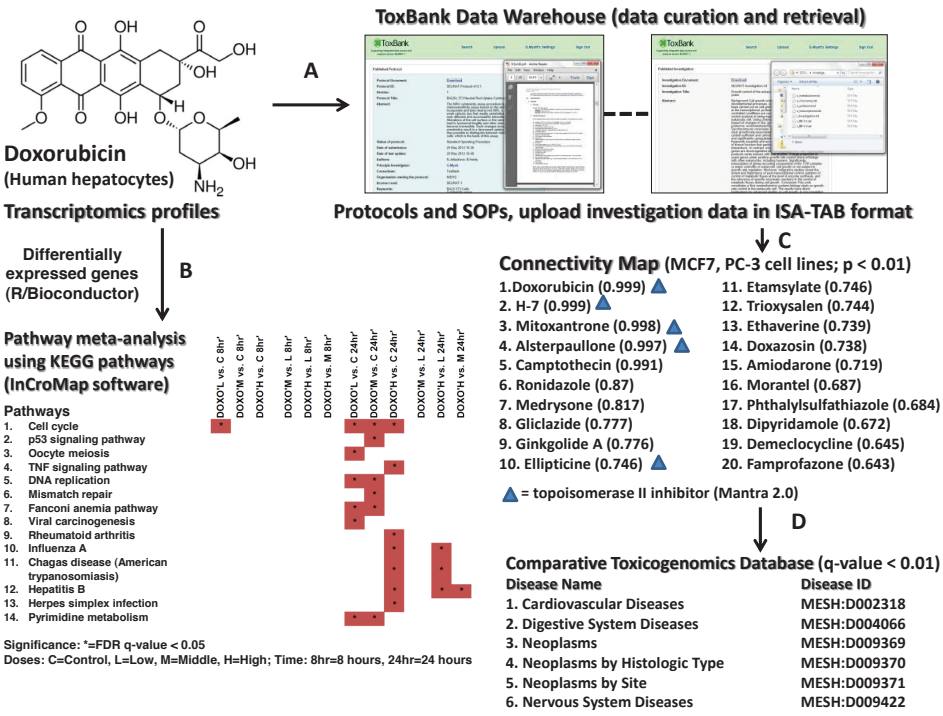

Figure 4.42 *A. Transcriptomics profiles of human hepatocytes treated with a compound of interest, e.g. doxorubicin, from the Open TG-GATEs repository are curated and deposited into the ToxBank data warehouse in ISA-TAB format. B. Differentially expressed genes can be extracted for different treatment concentrations relative the control (see legend). Kyoto Encyclopedia of Genes and Genomes pathway analyses depict molecular pathways influenced by the treatments. C. Top 100 up- and down-regulated genes (8 hours' time point, 10 μM concentrations) allows for connectivity mapping to genomic profiles of other agents with similar modes-of-action. Analysis with Mantra 2.0 implicates broad association to topoisomerase inhibitors. D. Analysis of the top 20 connectivity-retrieved agents from the Comparative Toxicogenomics Database generates hypotheses about the disease association; cardiovascular disease is the primary indication, being a known side effect of doxorubicin treatment (source: Kohonen et al., 2014; reprinted with permission).*

Gold Compound '-omics' Data

Pre-processing, normalisation, curation and differential expression analysis was carried out for the **SEURAT-1** Gold compounds. Results were submitted to the ToxBank data warehouse in ISA-TAB format (*Figure 4.42A*; *Sansone et al., 2010; Kohonen et al., 2013*). Open TG-GATEs human *in vitro* liver data for 158 compounds were downloaded via file transfer protocol from the NIBI website (http://dbarchive.biosciencedbc.jp/en/open-tggates/download.

html, last modified 27.3.2012). Of the 158 compounds in the human *in vitro* liver dataset, fourteen overlap with **SEURAT-1** Gold Compounds (see *Table 4.11*). The list of chemicals includes reactive compounds (e.g., acetaminophen, CCl_4), mitochondrial disruptors (e.g., rotenone), promiscuous binders (e.g., valproic acid, amiadarone), nuclear hormone receptor ligands (e.g., tamoxifen, WY14643), selective binders (e.g. fluoxetine) and cardiotoxins (e.g., doxorubicin, nifedipine). Adverse events of interest that are represented include cytotoxicity, fibrosis, steatosis, cholestasis and phospholipidosis. Detailed information about most of the compounds listed in *Table 4.11* can be found in the online-wiki (exception: WY14643; ToxBank, 2014).

Table 4.11 *Identities and modes-of-action of 14* ***SEURAT-1*** *Gold Compounds.*

	Hepatotoxins	
Toxicant	**Initiating Mechanism**	**Adverse Event of Interest**
	Reactive Molecules	
Acetaminophen	Non-selective thiol reagent	Cytotoxicity
Allyl alcohol	Selective thiol reagent, energy source	Fibrosis
Carbon tetrachloride (CCl_4)	Free radical generator	Steatosis, fibrosis
Aflatoxin B1	Lysine reagent	Apoptosis
	Mitochondrial Disruption	
Rotenone	Inhibition of complex I	Cytotoxicity
	Promiscuous Binding	
Valproic Acid	Membrane disruption, inhibition of fatty acid beta-oxidation	Steatosis
Chlorpromazine	Membrane disruption	Cholestasis
Amiodarone	Phospholipid binding, membrane disruption, inhibition of fatty acid beta-oxidation	Phospholipidosis, steatosis
	Selective Binding	
Fluoxetine	Phospholipid binding	Phospholipidosis
	Nuclear Hormone Receptor Ligands	
Rifampicin	PXR agonist	Negative control, steatosis
WY14643	PPAR-α agonist	Lipid metabolism disruption, proliferation
Tamoxifen	ER modulator	Epigenetics
	Cardiotoxins	
Doxorubicin	Topoisomerase inhibitor, redox cycling	Repeated dose organ failure
Nifedipine	L-type Ca-channel antagonist	Cell phenotyping

For all compounds, data for multiple time points (2 hours, 8 hours and 24 hours), concentrations (low, middle and high) and 2605 gene expression arrays were downloaded. The high concentration for this dataset was chosen to correspond with a reduction of lactate dehydrogenase activity by 10% or alternatively based on solubility in DMSO. The low, middle and high concentrations represented a fivefold dilution series (1:5:25 dilution), although considerations such as solubility necessitated deviations from the norm. The concentration ranges of the compounds in rat and human hepatocytes are reported in *Table 4.12*.

Table 4.12 *Doses of compounds within the study (rat and human hepatocyte in vitro data).*

Compound	Vehicle	Dose in rat hepatocyte (μM)			Dose in human hepatocyte (μM)		
		Low	Middle	High	Low	Middle	High
acetaminophen	medium	1000	3000	10000	200	1000	5000
carbon tetrachloride	DMSO	1000	3000	10000	300	1500	7500
valproic acid	medium	400	2000	10000	200	1000	5000
rifampicin	DMSO	2.8	14	70	2.8	14	70
allyl alcohol	medium	0.8	4	20	2.8	14	70
chlorpromazine	DMSO	0.8	4	20	0.8	4	20
WY-14643	DMSO	8	40	200	6	30	150
amiodarone	DMSO	0.28	1.4	7	0.28	1.4	7
tamoxifen	DMSO	0.12	0.6	3	NoData	5	25
nifedipine	DMSO	10	50	250	NoData	30	150
doxorubicin	0.5% DMSO	0.08	0.4	2	0.4	2	10
rotenone	medium	NoData	NoData	NoData	0.08	0.4	2
fluoxetine hydrochloride	DMSO	NoData	NoData	NoData	4	8	20
aflatoxin B1	DMSO	NoData	NoData	NoData	0.24	1.2	6

Files from Open TG-GATEs were processed individually using R scripts (version 3.0.2, 2013-09-25) and Bioconductor (version 2.13). Raw data files (.cel format) and phenotypic- and treatment-associated data were extracted (see *Table 4.13* for descriptions of terms in the data), and read into Bioconductor eSet data structures (*Gentleman et al., 2004*). Normalisation was carried out using the simpleaffy_2.38.0 package, the Robust Microarray Analysis (RMA) method (gcrma_2.34.0) and a custom .cdf file (hgu133plus2hsensgcdf_17.1.0), which maps Affymetrix HGU133Plus2 microarray probes to the most recent version of the human genome based on Ensembl gene models. The database contents were then formatted as R/Bioconductor eSet objects for further data mining.

Table 4.13 *Descriptions of the fields in the TG-GATEs gene expression file.*

Field name	Description
BARCODE	Barcode assigned to each GeneChip in order to identify it. Barcode matches CEL file name without extension.
ARR_DESIGN	In this project, HG-U133_Plus_2 was used for human and Rat230_2 was used for rat.
EXP_ID	ID assigned to each test which can be identified by a combination of COMPOUND_NAME, SPECIES, EXP_TEST_TYPE, and SINGLE_REPEAT_TYPE. IDs for *in vivo* tests are assigned from #0040. IDs for *in vitro* tests are assigned from #5000.
GROUP_ID	ID assigned to each group that can be identified by a combination of DOSE_LEVEL and SACRIFICE_PERIOD. IDs are in double digits (e.g. 01, 16).
INDIVIDUAL_ID	ID assigned to each individual/sample within a group. IDs are in single digit.
ORGAN	Organ evaluated in tests (liver or kidney).
MATERIAL_ID	ID assigned to each tissue section that was used to acquire gene expression data. IDs are in a single letter.
COMPOUND_NAME	Compound name.
COMPOUND_ABBREVIATION	Abbreviated compound name.
COMPOUND_NO	Number assigned to each compound. Numbers are not in serial order.
SPECIES	Species.
EXP_TEST_TYPE	Type of test. *In vivo* or *in vitro* test.
SINGLE_REPEAT_TYPE	Type of *in vivo* test (Single-dose test or 28-day repeat-dose test).
SEX_TYPE	Gender (male or female).
STRAIN_TYPE	Rat strain used with *in vivo* tests.
ADMINISTRATION_ROUTE_TYPE	Administration route (not relevant for *in vitro* tests).
ANIMAL_AGE	Age of animal (weeks). Only rats at 6 weeks of age were used.
SACRIFICE_PERIOD	Sampling time or period (incubation time for *in vitro* tests).
DOSE	Dose.
DOSE_UNIT	Unit of dose.
DOSE_LEVEL	Dose level. The ascending order of the levels is Control, Low, Middle, High.
Characteristics [DNA%]	Cell viability as measured by percentage of DNA.
Characteristics [LDH%]	Cell viability as measured by the Lactate Dehydrogenase assay.

The Open TG-GATEs human *in vitro* liver data was then investigated for batch effects. Principal Component Analysis (PCA, made4_1.36.0) and cluster analysis (Partitioning Around Medoids [PAM] method in cluster_1.14.4) of the data (after quantile normalisation over the entire pre-normalised dataset, limma_3.18.4) shows that three distinct batches exist in the data (*Figure 4.43*). However, these batches do not correspond with any of the experimental factors (data not shown) and the authors were not able to verify the origin of the batches either.

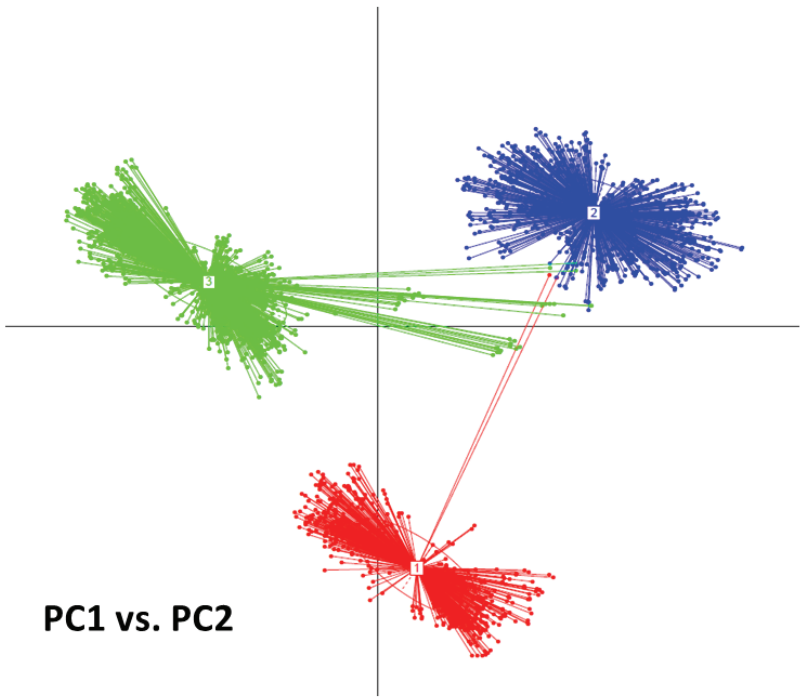

Figure 4.43 *Principal component analysis of the processed data reveals three distinct batches.*

The batches were first modelled and removed using the R/Bioconductor package sva_3.8.0 function 'ComBat', with the PAM-derived clusters as the batches. This enabled determination of the effect of cell culture growth time or rate on the overall expression levels in the data, which seem to fall on the first principal component (PC1). *Figure 4.44* indicates that the difference in overall expression between 2 hours and 8 hours is fairly small but becomes clearly distinguishable at 24 hours' time. After further analysis it was determined that the batches can be best normalised by taking ratios of treatment versus control for each compound, since every measurement of each compound is part of the same batch.

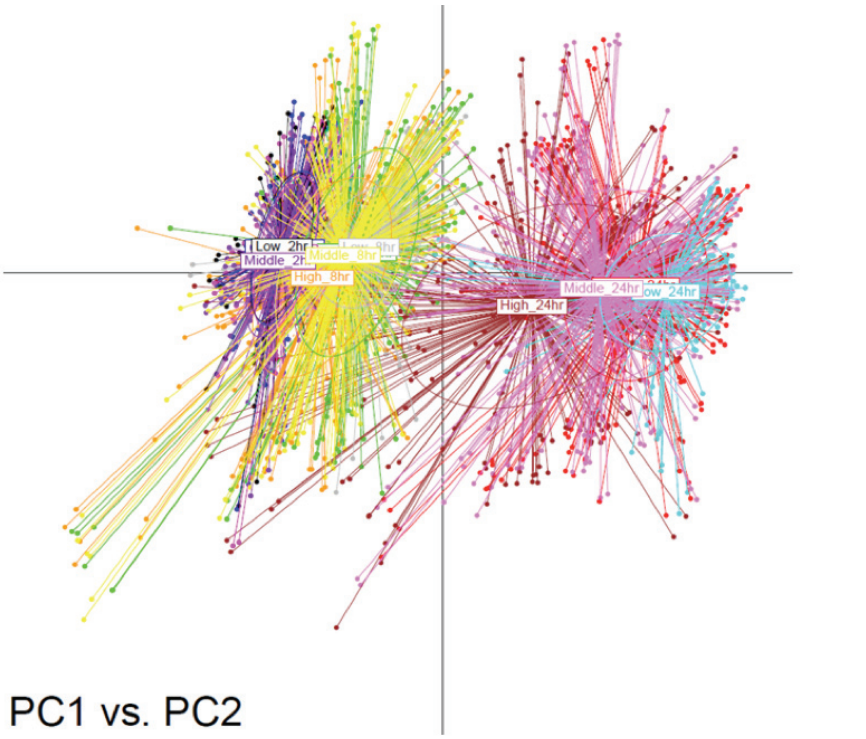

Figure 4.44 *Principal components analysis of batch effect normalised human hepatocytes treated with 158 compounds (2605 treatments) in three different concentrations (low, middle, high) and at different time points (2 hr, 8 hr and 24 hr). The expression values separate out by treatment time (2 hr and 8 hr versus 24 hr) as well as concentration (high 24 hr is distinct from other treatments at the 24 hr time point).*

The data for the 14 **SEURAT-1** Gold Compounds were then extracted from the dataset and differential expression analysis of each treatment was performed with respect to its closest control. The dataset contains two biological replicates for each treatment and control, so the empirical-Bayes variance shrinkage was employed from the limma_3.18.4 package to better estimate variance. Additionally, the mean-variance relationship was estimated by setting the trend=TRUE in the 'eBayes' function; the 'array weights' function was used to down-weight low quality arrays in the analysis.

Overall, there are 31,717 differential expression results with 14 compounds from the 45 comparisons that produced more than ten differentially expressed genes as a result. The threshold of differential expression was set to a 1.5 fold change (on the normal scale) and the multiple testing-corrected q-value needed to be below 0.05. The numbers of differentially expressed genes vary greatly by compound, by dose and time. High dose and 24-hour time point each had about two to three times as many results as the middle dose and 8-hour

time points, respectively (see *Figure 4.45A*). *Figure 4.45B* shows numbers of differentially expressed genes by compound (indicated by line colour) and by time (as indicated by line type). Doxorubicin had the largest amount of differentially expressed genes of all the **SEURAT-1** Gold Compounds, with almost 5000 differentially expressed genes obtained at the high dose and 24-hours (see *Figure 4.45B*). Based on this, doxorubicin was further investigated by applying pathway analysis to the differentially expressed genes (see below).

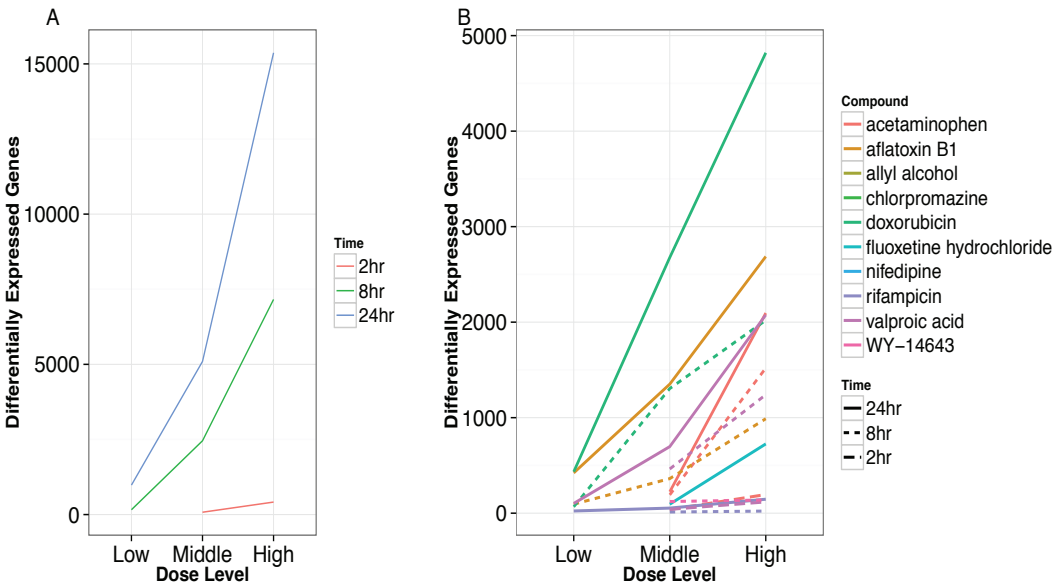

Figure 4.45 *Variation of the numbers of differentially expressed genes by dose and time (A) and by compound (indicated by line colour) and by time (as indicated by line type) (B).*

Gold Compound Meta and Pathway Analysis

Pathway activation can be studied using open source tools or commercial tools, such as the Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com) or the freely available InCroMap tool (http://www.ra.cs.uni-tuebingen.de/software/InCroMAP; *Wrzodek et al., 2013*). For mechanistic grouping of compounds, various bioinformatics techniques can sort the compounds into clusters by gene or pathway activation level (*Afshari, 2012; Kanehisa et al., 2014*). Network analysis of gene and protein activities may then identify upstream regulators, regulatory nodes or key regulator genes from the data, potentially constituting genomic signatures or biomarkers of toxicity (*Shi et al, 2010; Afshari, 2012*).

We developed and tested the following meta- and pathway analysis strategy for enriched '-omics' analysis of data on a selected reference compound (*Figure 4.42B*):

1. Evaluate the time- and dose-dependent processed '-omics' and functional data e.g. TX (C,t) for transcriptomics, PX (C,t) for proteomics, MX (C,t), and functional data for different functional experiments: F_1(C,t), F_2(C,t) etc. for the test compound;

2. Determine the set of statistically significant differentially expressed genes and P and Q values;

3. Use background gene-pathway knowledge (Kegg Pathway, GO Ontology) to enrich the toxicogenomic data sets of differentially expressed genes to determine the set of statistically significant pathways based on Q values; visualise the data against these pathways (e.g. using the InCroMap software);

4. Use background knowledge on chemical-biological interactions to enrich the toxicogenomic data. The strategy we explored was to enrich the toxicogenomic data using gene targets obtained from read-across of all PubChem data for positive assay results for similar compounds (e.g. using the read-across procedure described above, Tanimoto greater than 0.9), and repeat the enrichment and visualisation described in step 3;

5. Examine additional enrichment scenarios involving the combined addition of other data sets prepared in step 1 and repeat the enrichment and visualisation described in step 3;

6. Compare and interpret the results.

We applied this approach to the toxicogenomic data for TG-GATEs compounds and, in the following, describe the results for doxorubicin enriched by background knowledge from read-across of PubChem data and KEGG pathways. We use the following nomenclature here:

L0, L8, L24 = Signals at 0, 8 and 24 hours at low dosing concentration;

M0, M8, M24 = Signals at 0, 8 and 24 hours at medium dosing concentration;

H0, H8, H24 = Signals at 0, 8 and 24 hours at high dosing concentration;

Xt1Yt2 = Signal enrichment at X dosing concentration at time t1 relative to Y dosing concentration at time t2.

Examples:

H24L24 = Comparison of signals at high concentration relative to low concentration at 24 hours;

H24H8 = Comparison of signals at high concentration at 24 hours relative to 8 hours.

Background mechanistic information: Doxorubicin toxicity is initiated by oxidative damage associated both with the hydroquinone moiety and with iron complexes of the parent

compound. The major metabolic product is more toxic than the parent, but metabolism is not a requirement for toxicity. Doxorubicin intercalates with DNA (*Figure 4.46*) and thus causes direct damage to DNA as well as to proteins. Toxicity is both acute and chronic and is life-threatening.

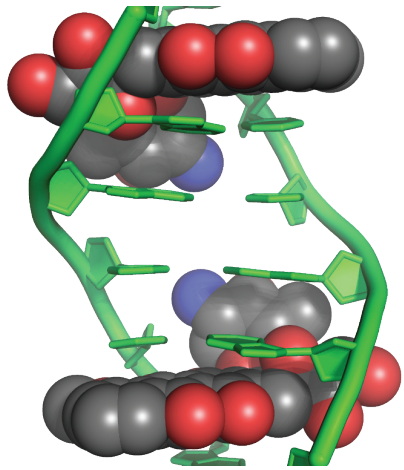

Figure 4.46 *Intercalation of doxorubicin with DNA (source: www.wikipedia.org).*

The underlying mechanism of doxorubicin cardiomyopathy is oxidative damage. The effects of this oxidative reactivity include direct DNA damage, accumulation of mitochondrial DNA mutations, alterations in calcium handling, proteolysis of titin, and dysregulation of cardiac transcription factors. Damage is selective, but not exclusive, for DNA because of intercalation of doxorubicin. Oxidative reactivity is generated via hydroquinone-quinone redox cycling from the hydroquinone moiety of the parent drug and via complexation of the drug with iron. The relative importance of these two pathways is not fully established (*Ewer & Ewer, 2010*).

Low dosing concentration: At low dosing and early time (L8) only cell cycle pathways are showing significant disturbance (data not shown). At longer times (L24) there is a significant increase in perturbed pathways such as DNA replication. Pathways ‘drug metabolism’ and ‘ALS’, which are seen as significant interactions in PubChem assays, are not observed as significant in the DEG-enriched pathways. One pathway at L24 conditions was found to be significantly enriched with regards to PubChem pathway interactions: Fanconi anemia is an inherited genomic instability disorder, caused by mutations in genes regulating replication-dependent removal of interstrand DNA crosslinks. The Fanconi anemia pathway (*Figure 4.47*) is thought to coordinate a complex mechanism that enlists elements of three classic DNA repair pathways, namely homologous recombination, nucleotide excision repair, and mutagenic translesion synthesis, in response to genotoxic insults. To this end, the Fanconi Anemia pathway employs a unique nuclear protein complex that ubiquitinates FANCD2

and FANCI, leading to formation of DNA repair structures (*Moldovan & D'Andrea, 2009*). This pathway signal can thus be interpreted as indicating DNA damage even at low dose concentrations.

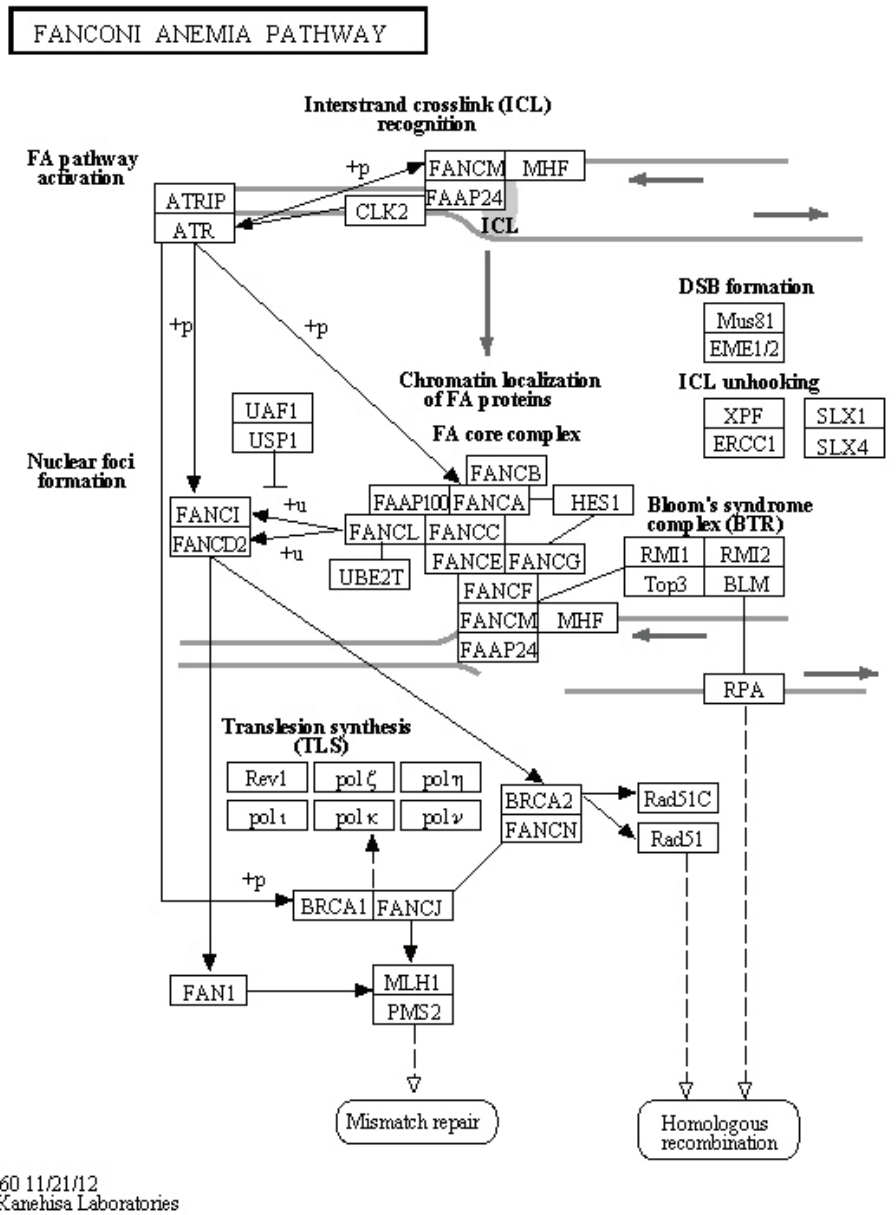

Figure 4.47 *KEGG Fanconi anemia pathway description (homo sapiens; source: http://www.genome.jp/dbget-bin/www_bget?map03460).*

Medium dosing concentration: Results at medium doses (M24) shows an increase in interactions with p53 signalling, Fanconi anemia and mismatch repair pathways (data not shown). Two pathways at M24 conditions were found to be significantly enriched with regards to PubChem pathway interactions: the Fanconi anemia pathway observed at low doses and additionally the neuroactive ligand-receptor interaction based on the GALR2 interaction.

High dosing concentration: Results at high dosing and shorter times (H8) shows a large increase and difference in perturbed pathways as compared with low and medium doses with the TNF signalling pathway showing the highest significance and indicating the initiation of cell death. At high doses and longer times (H24) disease pathways such as Influenza A and Hepatitis B show increased disturbance. Several pathways at H24 conditions were found to be significantly enriched with regards to PubChem pathway interactions observed for positive interactions for doxorubicin only: NF-kappa B signalling pathway, PI3K-Akt signalling pathway and apoptosis. The NF-kappa B signalling pathway and its interactions are shown as an example in *Figure 4.48.*

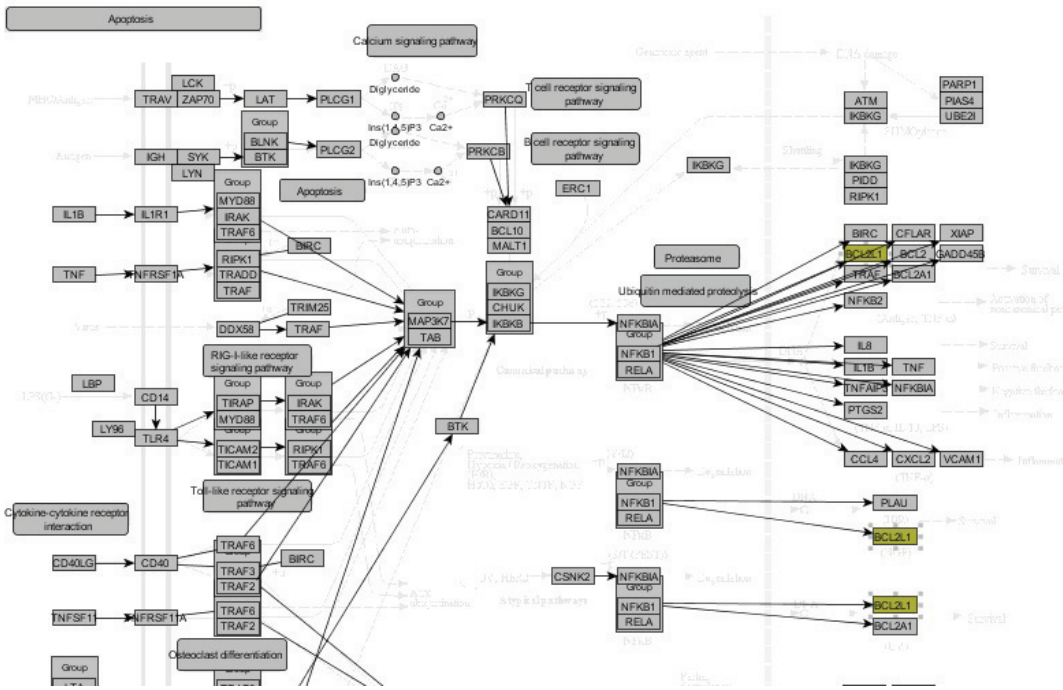

Figure 4.48 *Enriched NF-kappa B signalling pathway and its interactions for doxorubicin (H24L24).*

When the H24 results were also enriched for similar compounds by PubChem read-across, significant pathways included toxoplasmosis, PI3K Akt, measles, influenza A, Jak-STAT and endoplasmic reticulum protein processing (data not shown).

Connectivity Mapping to discover Modes-of-Action and Biologically Similar Chemicals

Connectivity mapping has been suggested as a very useful tool for toxicity testing and for facilitating biological read-across (*Lamb, 2007; Smalley et al., 2010; Kohonen et al. 2014*). The Connectivity Map (CMap) database has thousands of genome-wide expression profiles of chemical perturbations, mainly using US Food and Drug Administration approved drugs, on three cancer cell lines. Investigators searching the CMap enter over- and under expressed genes into the search engine, ranking chemotherapeutic agents on whether they regulate the same genes, either in an opposite or similar fashion. The Mode of Action by NeTwoRk Analysis Mantra 2.0 database has clustered the CMap database and annotated the mode-of-action of each compound, enabling determination of an unknown compound's mode-of-action by referring to the neighbouring compounds in the network (*Carrella et al., 2014*). Connectivity mapping has been implemented for the TG-GATEs dataset in the liver toxicity map service; the Toxygates interface to the TG-GATEs data also enables ranking of compounds based on the genes that they regulate (for a review of databases for toxicogenomics see *Kohonen et al., 2014*).

Gene expression data profiles of doxorubicin from the TG-GATES database were next analysed using the Connectivity Map (CMAP) database to identify similar chemicals for read-across and to characterise the chemical's mode-of-action (*Figure 4.42C*). Interestingly an analysis with the most significantly altered genes in the CMap service identifies doxorubicin itself and other topoisomerase inhibitors such as mitoxantrone and camptothecin. Anthracyclines and related substances (ATC code L01DB01) are also enriched as a class, showing that commonly used cancer cell models and primary liver cells can have very similar profiles. Analysis of the CMap enriched compounds using the Mantra 2.0 tool likewise indicates many of the identified connections are topoisomerase II inhibitor compounds (see *Figure 4.42C* for mode-of-action predictions from mantra 2.0).

Chemical Set Enrichment Analysis to Develop Hypotheses for Toxicity by Read-Accross

The Comparative Toxicogenomics Database (CTD) connects chemicals with gene expression changes as well as with diseases, which can be used to give information on toxicity associations of chemicals at the organ or organism levels (*Davis et al., 2013*). CTD enables chemical set enrichment analysis whereby a list of chemicals is entered into the service and enrichment

analysis is performed based on the gene-associations extracted from that list. Analysis of the top 20 chemicals from the CMap using associations to the CTD's MEDIC disease vocabulary points to "Cardiovascular Diseases" as the most strongly enriched disease (*Figure 4.42D*). Therefore the results are in line with doxorubicin causing cardiomyopathy and being a topoisomerase inhibitor that intercalates with DNA and induces oxidative DNA damage (*Ewer & Ewer, 2010*). Thus, publicly available tools and databases help generate a correct hypothesis of systemic toxicity and define the mode-of-action of a toxicant.

Conclusion

Toxicity assessment can be seen both as a data-driven activity and concept-driven activity. Connectivity mapping with gene expression or cell-based HTS data is an example of data-driven activity, as is QSAR modelling (*Smalley et al., 2010; Kohonen et al., 2014*). Differently, the description of molecular initiating events and key events that lead to an adverse outcome is a concept-driven activity that facilitates evaluation of evidence for toxicity (*Vinken, 2013*). The AOP Wiki currently developed under the Organisation for Economic Co-operation and Development (OECD) guidelines will allow users to cooperate in documenting and evaluating information underlying AOPs. Integrating mechanistic understanding with data from HTS and toxicogenomics efforts will facilitate AOP development and use so that compounds can be assigned to various classes based on the cellular toxicity pathway activities that they trigger.

On-going reductions in costs for sequencing and multiplexing will make the use of high-content information technologies, especially transcriptomics, increasingly attractive. In parallel, the relative cost, and complexity, of data interpretation is instead bound to increase (*Sboner et al., 2011*). The **ToxBank** consortium is facilitating the development of well-standardised and documented bioinformatics workflows that are key requirements for integration of various '-omics', HTS and chemical structural descriptor data. Solutions to standardisation of data and meta-data descriptions sufficiently fit for biomarker development and modelling come from implementing standardised file formats such as ISA-TAB, ontologies, e.g., for experimental factors, chemical structural descriptors, and also standard operating procedures for accurate models and classifiers of toxicity (*Sansone et al., 2010, Kohonen et al., 2013*). Constituting a useful example of such efforts, OpenTox has provided an extensive specification for an open interoperable standards-based predictive toxicology framework involving components for data, algorithms, compounds, biological features, models, validation and reporting which may be used to develop such workflows (*Hardy et al., 2010*).

4.8.4 Innovation

The ToxBank Data Warehouse has continued to evolve and new innovations include the creation of an investigation 'dashboard' to visualise multiple experiments as well as to easily export

raw and processed data, enabling integration with general bioinformatics tools for analysis and enrichment along with general analysis, visualisation, modelling, and data mining tools to support understanding the results and performing a meta analysis of the data. To support this integration and analysis, a new standard to harmonise processed data fields generated from transcriptomics, proteomics, metabolomics, metabolic flux, epigenetics, miRNA, qRT-PCR, kinetics, and *in vitro* and *in vivo* dose response experiments has been proposed. In addition, precise searching for chemical structures has been added to the ToxBank Data Warehouse (exact, substructure, and similarity) to support read across and information look-up.

4.8.5 Cross-Cluster Cooperation

Right at the start of **SEURAT-1** Research Initiative, the first two **SEURAT-1** cross-cluster working groups were established by **ToxBank**: the Data Analysis Working Group (DAWG) and the Gold Compound Working Group (GCWG). DAWG meetings and communications discussed the expected data analysis requirements of the cluster. The GCWG meetings were held to finalise the list of standard reference compounds to be used in the **SEURAT-1** case studies. The cross-cluster working group approach proved particularly successful and was adopted by COACH and expanded into other areas during 2012 as a key organisational structure for cluster activities (see also section 4.11.2). The working group activities provided valuable background information and interactions that aided the development of the warehouse design.

ToxBank has continued to collaborate with DETECTIVE, NOTOX, *HeMiBio*, and *SCR&Tox* to create ISA-tab formatted investigations and protocols to upload into the ToxBank Data Warehouse as well on the analysis of the data. **ToxBank** has also collaborated with COSMOS to provide access to the COSMOS data. A single structure search from the ToxBank Data Warehouse will return matching chemicals with integrated records that have been uploaded to **ToxBank** alongside COSMOS database records.

4.8.6 Expected Progress within the Fourth Year

A number of extensions to the **ToxBank** Data Warehouse will become available in 2014. These include the ability to upload case study templates, as well as related reports and publications. It will also be possible to register an investigation and upload preliminary unformatted data. A new upload status dashboard will summarise all reports, investigations, and protocols that have been uploaded and will be available in 2014. The information will be organised wherever possible by the different **SEURAT-1** case studies that the data or any of the documents support. This dashboard will also highlight the status of the upload. In 2014, a new biomaterials search capability will come online to support searching for important genes, proteins, and cell lines. The ToxBank Data Warehouse will also integrate information from the Tox21 and ToxCast initiatives (*Judson et al., 2014*).

4.8.7 Future Perspectives

The **ToxBank** project establishes critical infrastructure and services for 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 (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, potentially use and report on alternatives, wherever possible. Therefore, industry is another major target stakeholder of our infrastructure as industry-standard resource facilities such as **ToxBank** are required for safety assessment activity. In particular, SMEs will be challenged by regulations as they frequently do not have in-house tools and knowledge resources for the assessment work. **ToxBank** should also have beneficial impact on Cosmetics Europe and other organisations affected by the Cosmetics Directive. This directive places strong legislative 3Rs requirements on consumer product companies as all systemic toxicity animal experiments were to be replaced, starting in 2013.

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

COACH

Sara Vinklatova, Emmanuelle Da Silva, Bruno Cucinelli

4.9.1 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 aims of **COACH** are 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 centralised scientific administration to the **SEURAT-1** Research Initiative (the '**COACH** Office'), organising cluster-level interactions and activities and being the main cluster-level entry point at the for all organisations, including funding organisations, such as the European Commission and Cosmetics Europe, as well as any external organisation looking to liaise with the **SEURAT-1** Research Initiative (*Figure 4.49*).

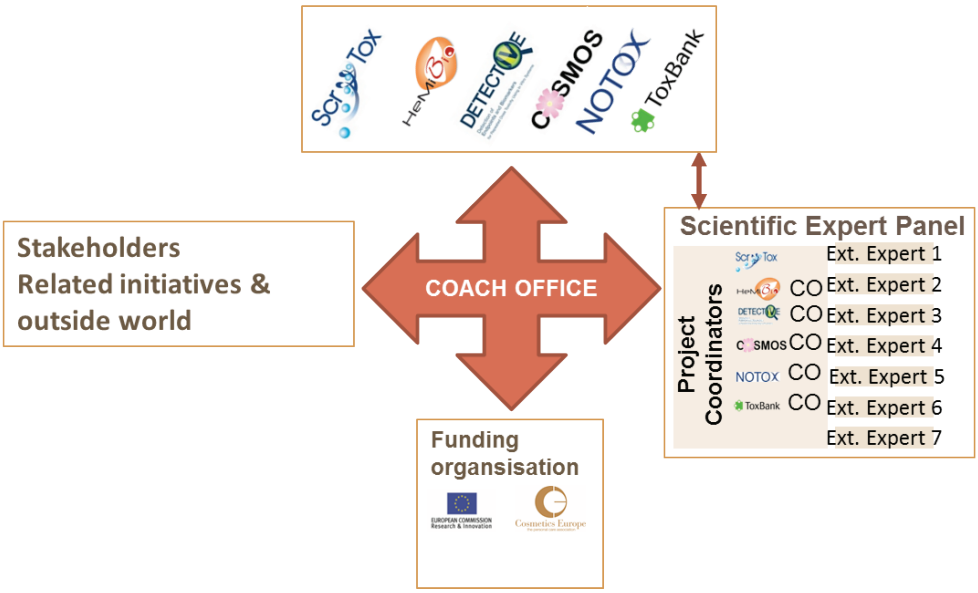

Figure 4.49 *The* ***COACH*** *Office as the central contact for cluster-level activities.*

Each of the seven projects of the **SEURAT-1** Research Initiative 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. A common hearing session with independent reviewers was then organised by the European Commission and Cosmetics Europe in early 2013.

The second 18-month work period finished in December 2013. In order to allow the reviewers appointed by the European Commission and Cosmetics Europe to have opportunities for more direct exchanges with the **SEURAT-1** partners, and to get a concrete view of the cluster level cooperation activities, the Project Officer of the European Commission and **COACH** decided to invite the reviewers to the **SEURAT-1** Annual Meeting in February 2014.

The following sections highlight some important achievements of the first, second and beginning of the third periods (illustrated in *Figure 4.50*).

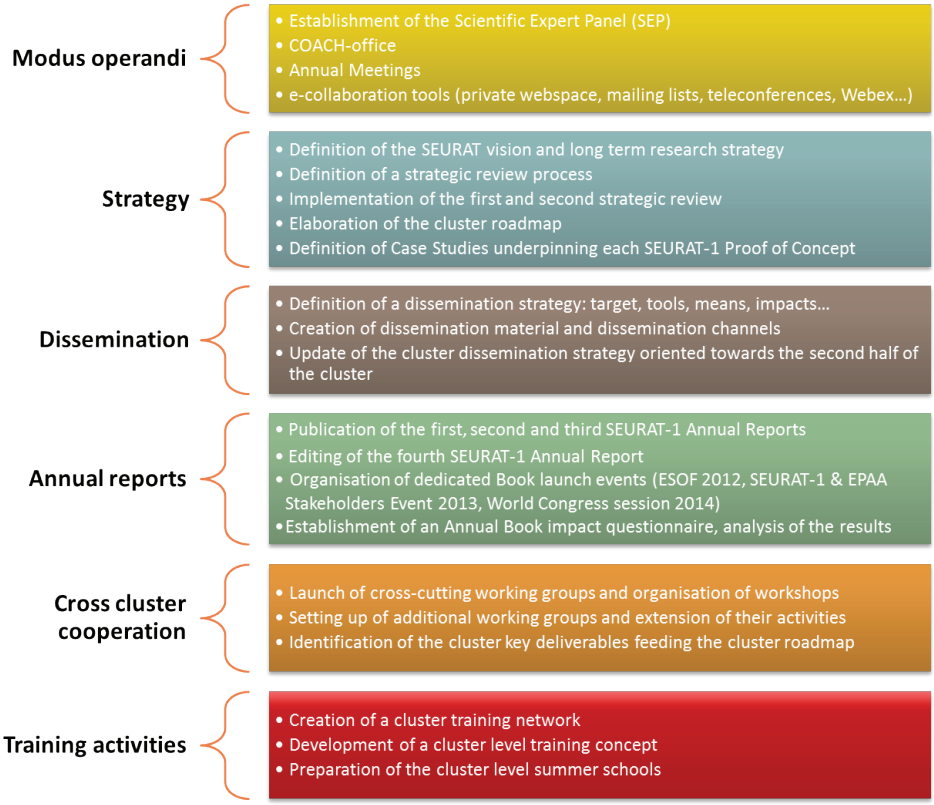

Figure 4.50 *Main cluster-level achievements of the* ***SEURAT-1*** *Research Initiative since the launch of the initiative.*

4.9.2 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 the collaboration, partners need to establish the methods, means and common references that allow them to organise the collaboration in the most efficient and productive 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 that work towards a common aim. **COACH** played a key role in this specific context and thus the achievements of the first three years 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 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 Tuebingen 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, the global research target of **SEURAT-1** possible follow-up activities.

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 the Joint Research Centre and consists of two main components: (i) a SWOT analysis questionnaire 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 cluster coordinators and the SEP members as well as Cosmetics Europe Advisory Board members. Feedback was collected and summarised, and was then further discussed by the SEP to identify actions that would improve cluster interactions and achieve a high-level outcome. This exercise is repeated on an annual basis and, thus, provides the SEP with a tool to understand whether improvement measures have been successful.

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. The majority of SWOT analysis 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 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 cluster coordinators were presented in a subsequent SEP meeting in November 2012; the finalised second strategic review was presented at the SEP meeting in June 2013. Further details, including the second **SEURAT-1** roadmap, are given in section 4.11.1 of this Annual Report.

Updating the strategic review is performed on a regular basis and its results are reported formally at each SEP meeting. This allows the SEP members to identify potential gaps and weaknesses. Dedicated discussions on the progress and possible improvements of cross-cluster interactions on the basis of the updated roadmap are also organised.

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), Tox21/ToxCast (research programmes of the US Environmental Protection Agency) and ESTIV (European Society of Toxicology In Vitro). More details about related international research programmes are summarised in section 5.2.

The organisation of the **SEURAT-1** & ESTIV Joint Summer School illustrates the role of **COACH** in supporting collaborations between **SEURAT-1** and related initiatives. The Summer School was organised as part of the training strategy by **COACH** partner ARTTIC, and took place on 8-10 June 2014 in Amsterdam. It brought together *in vitro* and *in silico* toxicologists from many different countries, representing academia, industry and regulatory bodies, as well as **SEURAT-1** young scientists who showcased their work within **SEURAT-1**. More details on this fruitful collaboration can be found in section 4.12, which focuses on 'Training and Outreach'.

The launch of the Annual Report also provides opportunities for deepening relationships with other international activities. The third Annual Report was presented during the **SEURAT-1** & EPAA Stakeholders Event, which took place in Brussels on 5 September 2013. This book launch event reached out to policy makers, regulators, industry, animal welfare groups and the general public, and provided a view on the progress made so far by the consortium. The latest success stories and highlights at the cluster level were presented in a practical and accessible manner and the third Annual Report was officially launched.

The launch of the fourth Annual Report will occur at the 9th World Congress on Alternatives and Animal Use in the Life Sciences, to be held in Prague in August 2014. **COACH** is currently preparing the **SEURAT-1** corner, hosted at the Joint Research Centre booth, where all **SEURAT-1** publicity material (leaflets, posters, USB sticks, all volumes of the Annual Reports, etc.) will be made available. The stand will also host miscellaneous activities, such as interviews, short sessions on various topics covering the **SEURAT-1** research domain, videos and other activities underpinning the book launch.

4.9.3 Facilitating Exchanges between SEURAT-1 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** established 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 **SEURAT-1** private workspace is accessible by 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 contact between cluster participants. The first two Annual Meetings (March 2011 and February 2012) were organised with the following structure: (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 and fourth Annual Meetings (March 2013 and February 2014) were organised differently, in order to adapt to the evolving cross-cluster cooperation needs of the initiative and address the increasing need to work on the **SEURAT-1** Case Studies (see chapter 3). All the projects are now advanced in their work programmes and a number of new methodologies and tools for mechanism-based toxicology were unveiled at the fourth **SEURAT-1** Annual Meeting in February 2014. This Annual Meeting also provided a forum to discuss the carefully designed Proof-of-Concept (PoC) case studies through dedicated presentations, poster and breakout sessions. It has become tradition at the Annual Meetings that 'Excellent Poster Awards' are presented to selected **SEURAT-1** young scientists. In Barcelona four were awarded: Sophie Teng *(*COSMOS*)*, Sofia Batista Leite *(HeMiBio)*, Dimitar Hristozov (COSMOS) and Scott Miller (ToxBank). Extended abstracts of their work are given in section 4.10.3).

Another important element of fostering collaborations between scientists in the different research projects is the organisation of cross-cluster working groups. A detailed overview of these working groups is given in section 4.11.2 and activity reports are presented in sections 4.11.3–4.11.8. To initiate and stimulate the working groups, **COACH** partners the Joint Research Centre and the University of Tübingen organised workshops during the Annual Meetings as breakout sessions; meetings at the Joint Research Centre in Ispra, Italy; and actively participated in other workshops, such as the Biokinetics Working Group meeting in Paris (details are given in the activity reports of the working groups). Following a proposal from the Joint Research Centre, each working group has a clearly defined scope and is coordinated by two co-leaders. Besides actively preparing workshops (see above), **COACH** supported these working groups in organisational matters, organised teleconferences as required and set up mailing lists and dedicated workspaces for each working group on the collaborative private web platform, in order to facilitate communication and collaboration among the working group members.

Based on the homogenised training concept and the establishment of a training task force composed of representatives from each of the projects, **COACH** organised the second cluster-level summer school on 8–10 June 2014 linked to the ESTIV2014 conference. The **SEURAT-1** part of the summer school covered mostly practical sessions (computer hands-on, soft skills sessions, demonstrations), whilst the ESTIV programme covered the theoretical topics. Section 4.12.1 provides further details on the training activities.

4.9.4 Information Dissemination

Ensuring good visibility of the research initiative is one of the key activities of **COACH**. To continue spreading information on **SEURAT-1**, its developments and results, publicity material was created in various formats and suitable dissemination channels have been set up and are described below.

Firstly, a consistent visual identity for **SEURAT-1** (logo, colours, layout of printed and electronic publicity material, website look and feel, etc.) had been developed in the outset of the initiative in collaboration with a professional design company.

Secondly, a variety of information dissemination support materials has been created and distributed, including: a first version of an information leaflet; a second version of the leaflet containing an embedded USB stick; **SEURAT-1**, COACH and Roadmap posters; a 'who's who' booklet, which is distributed at each Annual Meeting (also available online); and a standard PowerPoint presentation. A unique roll-up banner was created for the **SEURAT-1** & ESTIV Joint Summer School, and to support the next book launch at the '9th World Congress on Alternatives and Animal Use in the Life Sciences'.

In addition, the public website (www.seurat-1.eu) was kept up-to-date and extended with additional information. The website presents the research initiative, its background and aims, the cluster projects and partner organisations involved, and promotes research activities in the field of human safety assessment, in particular regarding alternatives to *in vivo* repeated dose systemic toxicity testing. Dedicated, regularly updated pages present related events, links, publications, job announcements, etc. The most recent updates include a brand new FAQ page, Annual Report questionnaire (live for one month) and mid-term update of the objectives.

The preparation of the first, second and third **SEURAT-1** Annual Reports was coordinated by the **COACH** partner University of Tübingen, who proposed the content structure and specified the 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 validation of the book's contents. The University of Tübingen collected, reviewed and edited the contributions while ARTTIC took care of the book layout in collaboration with a professional designer. The first Annual Report (*Schwarz & Gocht, 2011*) was successfully completed in September 2011. Online and printed copies of the second Annual Report (*Gocht & Schwarz, 2012*) were available in July 2012 and it was presented at the Euroscience Open Forum (ESOF). The third Annual Report (*Gocht & Schwarz, 2013*) was issued in July 2013 and officially launched at the **SEURAT-1** & EPAA Stakeholders Event in September 2013. Between 1,300 and 1,500 copies of each Annual Report are printed and distributed to individuals by regular post and at relevant conferences (further details on the event are given in section 4.12.3). Electronic copies of the Annual Reports are available for download from the **SEURAT-1** public website. Finally, all volumes of the Annual Report have been loaded onto **SEURAT-1** USB sticks; with the distribution of these means we are reaching even more of the target audience. A dedicated dissemination channel for the Annual Report was created in the form of a mailing list, containing over 700 postal addresses of scientists, experts and stakeholders in **SEURAT-1** research results.

The **COACH** partners are aware of how important promoting the objectives, approach and progress of **SEURAT-1** is at international conferences and workshops. Their active participation

to many of these events strongly contributed to increasing the visibility of **SEURAT-1** on the international scene, hence triggering further interest; this is largely illustrated by an increase in requests to receive the Annual Reports. The events where representatives of **SEURAT-1** were present are listed in section 4.12.3.

The 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, is the result of a dissemination plan prepared as a project internal working document by **COACH** in 2011. For the first time, it defined the dissemination objectives, the appropriate means required to reach the targets and led to a number of actions. The research community remains an important target for information dissemination, exchanges and collaboration but, as already reported in the last volume of this book, a need was identified last year 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. Consequently, a paper describing an updated dissemination strategy was prepared by **COACH** and presented during the SEP meeting on 24 June 2013. The dissemination strategy aims to define dissemination objectives, means and channels to better target the stakeholder groups and, accordingly, to establish a plan of appropriate concrete dissemination actions. This dissemination strategy is considered to be a living document that is reviewed in each SEP meeting. The main actions resulting from the strategy are to:

- Investigate the impact of the Annual Report (questionnaire set up via the public website);
- Strengthen links with EPAA (organisation of the **SEURAT-1** & EPAA Stakeholders Event);
- Strengthen links with regulators (currently negotiating with ECHA on common collaboration in 2015/2016);
- Continue to disseminate to the international scientific community (**SEURAT-1** increased its participation in major scientific events and **COACH** prepared a scientific paper to be published in a peer-reviewed journal);
- Strengthen links with animal welfare groups (close collaboration with several representatives was established; the concrete actions are being discussed).

The dissemination strategy is now the key reference for **SEURAT-1** dissemination activities and is regularly updated at each SEP meeting or teleconference.

4.9.5 Next Steps

The **COACH** action list currently includes the following work topics:

The second update of the strategic review of the cluster: **SEURAT-1** has advanced greatly

since the first issue of the cluster strategic review and the SWOT analysis. **COACH** now plans to prepare a new update of the SWOT analysis with the aim of presenting the outcome of its strategic review at the SEP meeting on 28 May 2014.

Workshop on read-across case study and teleconference on ab initio case study: The level 2 case studies work programme was defined over the course of the last year. Planning of the level 3 case studies, focusing on the application of **SEURAT-1** methods in the context of risk assessment, is now underway. **COACH** is actively supporting the Safety Assessment Working Group in their efforts. **COACH** provides infrastructure and organised: (i) teleconferences for discussing the scope of the ab initio safety assessment case study; and (ii) a workshop in Ispra, Italy (29–30 April 2014), hosted by **COACH** partner Joint Research Centre, to discuss the design of the read-across case study. This workshop was attended by external experts who showed great interest in participating on the planning of this particular case study (see also section 2.6). **COACH** is not only providing infrastructure, but is also actively involved in the ongoing discussions about the content of these case studies and their coordination with the activities regarding level 2 case studies (see chapter 3).

Liaison with related initiatives and communication to general public: 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 during the next Summer School and the book launch at the 9th World Congress on Alternatives and Animal Use in the Life Sciences. During these events **COACH** plans to increase the visibility of **SEURAT-1** by massive communication towards the target groups as defined in the dissemination strategy. This will be done by creating new publicity materials (roll-up banner, video documentation) and by inviting scientific journalists to participate in the events.

Organisation of the second cluster level Summer School: This event, mainly focusing on practical courses, took place in combination with the ESTIV2014 conference. **COACH** organised this important event. More information is already available in the chapter 'Training and Outreach' (see section 4.12.1); feedback and outcomes will 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. Since the **SEURAT-1** Research Initiative has passed the midpoint of its five-year duration, partners and stakeholders are thinking about the organisation of the next phase of the required long-term research work: What will be the scope of SEURAT-2? What forms of public-private partnership could be envisaged? 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 fourth year will also address the further development of the achievements made in the past periods, i.e., efficient operation of the Working Groups, support of the cross-cluster collaboration on the Proof-of-Concept case studies, preparation of the next training activities, and collaboration with related research initiatives and organisations.

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4.10 Project and Cluster Activities

4.10.1 Project Meetings

Mark Cronin, 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. Besides the Annual Meeting in early 2013 (reported in the last volume of this Annual Report) two face-to-face meetings have taken place in the third year; the first was the *SCR&Tox* six-monthly meeting held in Paris, France, on 7 June 2013. The discussion was focused on the work programme for the second phase of the project that is dedicated to the assay development and implementation at the industrial scale. The second meeting was held on 3–4 October 2013 in Stockholm, Sweden. Achievements within the different work-packages were presented in this meeting and discussion about the planning of the work programme was continued.

The fourth *SCR&Tox* annual meeting was held in London, England, on 13–14 February 2014. Representatives of all work-packages were present and decisions were made about which specific cell lines will be used for the development of the industrial prototype (work-package 4). Members of the external advisory board were also present. The **SEURAT-1** cluster-level was represented by Tilman Gocht, a member of the COACH consortium.

SCR&Tox consortium members have found the three-monthly web-conference meetings to be a fruitful resource for scientific discussion and risk management.

HeMiBio: For the purpose of efficient risk management, *HeMiBio* consortium holds face-to-face meetings every six months and web-conferences on a three-monthly basis. Three face-to-face meetings were held in the third year: The 30 months progress report meeting took place on 9–10 July 2013 in Berlin and the second *HeMiBio* Annual Consortium Meeting on 23–24 January 2014 in Leuven, Belgium. The discussions during these meetings focused on progress in the work-packages, the definition of the workplan for the next six months and input required from the **SEURAT-1** Research Initiative to be presented at the **SEURAT-1** Annual Meeting. In addition to these planned meetings, an extraordinary Executive Committee meeting was held on 13 September 2013, just before the second joint meeting with other **SEURAT-1** projects, to discuss specifically the progress in the fabrication of the flow-through bioreactor. The Annual Meeting was attended by members of the *HeMiBio* External Advisory Board who provided feedback on the last year's progress and advice on upcoming priorities. The **SEURAT-1** cluster level was again represented by Tilman Gocht, a member of the COACH consortium. A Winter School focusing on pluripotent stem cells and their differentiation into

different liver cell types was held just before the Annual Meeting on 21–22 January 2014 in Leuven, Belgium (a summary is given in chapter 4.12.1). Finally, the three-monthly web-conference has concentrated on the biological aspects of the project and in particular on cell engineering progress.

DETECTIVE: The third DETECTIVE General Assembly took place on 16 June 2013 during the DETECTIVE Summer School on in Slano, Croatia. A major progress in the resolution of technical and methodological issues was demonstrated. Furthermore, the integration of different datasets was initiated. The DETECTIVE advisory board attended the meeting. Further information about the summer school can be found in section 4.12.1. Several meetings were organised in the last year and addressed (i) the project roadmap; (ii) the three **SEURAT-1** proof-of-concept case studies with DETECTIVE involvement; and (iii) ongoing data integration for biomarker verification.

COSMOS: The Third COSMOS Annual General 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-led by COSMOS.

The six-monthly COSMOS General Assembly meeting was hosted by COSMOS partner the National Institute of Chemistry in Ljubljana, Slovenia on 9–10 September 2013. This meetig focused on ongoing work within the work packages, such as the input from QSAR models and metabolism prediction into physiologically-based pharmacokinetic (PBPK) models. The meeting also included preparations for the public launch of the COSMOS Database, training for the database structure curation using the COSMOS Data Entry System and hands-on testing of the COSMOS Space functionalities. New KNIME workflow functionalities and the WebPortal were presented. Scientific Advisory Board member Scott Boyer from AstraZeneca contributed an overview on the expectations of the project from the industry perspective as well as links to the eTox project. Further dissemination at upcoming conferences was planned.

At the end of the third project year, the fourth COSMOS Annual General Meeting took place in Barcelona, Spain on 3–4 February 2014, before COSMOS delegates joined the Fourth **SEURAT-1** Annual Meeting on 5–6 February 2014. The work status was presented and planning for the remaining two years of the COSMOS project was discussed, including COSMOS involvement in the different ongoing cross-cluster **SEURAT-1** case studies. Route-to-route extrapolation was a theme throughout the discussions in the different work-packages: for the

work on PBPK models, the modelling of skin permeability and the evaluation of the Threshold of Toxicological Concern (TTC) approach for cosmetics. Another common theme was the implementation of models into KNIME nodes and workflows. Developed KNIME workflows were demonstrated. Furthermore, the COSMOS Database webinar and contributions to the **COSMOS** symposium session at the 53rd Annual Meeting of the Society of Toxicology (SOT 2014) were planned.

In addition to these plenary meetings, COSMOS partners and work-packages held many additional meetings or teleconferences in small groups to discuss specific questions for the ongoing work. The **SEURAT-1** Biokinetics Working Group meeting on 24–25 September 2013 in Paris, France, hosted by COSMOS partner and Working Group leader the French National Institute for Environment and Risk, was also important for the further planning of COSMOS work on toxicokinetics in the safety assessment of cosmetic ingredients.

Furthermore, the ILSI-EU COSMOS TTC Expert Groups met frequently via teleconference or face-to-face, the latter, for example, in April 2013 in Brussels, Belgium and at the EUROTOX 2013 conference on 3 September 2013 in Interlaken, Switzerland. These discussions included approaches and progress regarding the development of the new COSMOS TTC dataset as well as the oral-to-dermal-extrapolation for the evaluation of the TTC approach to cosmetics-related substances.

NOTOX: The fifth NOTOX progress meeting took place on 3–4 September 2013 in Dortmund, Germany, and was hosted by the Leibniz Research Centre for Working Environment and Human Factors (IfADo). More than 30 scientists from the twelve partner institutions discussed the intermediate status of the project and also future collaborative work. Barry Hardy, the scientific coordinator of the ToxBank consortium, attended the meeting and provided NOTOX partners with valuable insights into the supporting integrated data analysis and servicing of alternative testing methods in toxicology.

The sixth progress meeting was hosted by Cambridge Cell Networks in Heidelberg, Germany, on 24–25 February 2014. Tilman Gocht from COACH and Cosmetics Europe representative Yeyejide Adeleye attended this meeting. Various decisions on future collaborative work and publications were made.

The first public NOTOX Satellite Meeting was organised during the 'European Society of Toxicology *In vitro*' (ESTIV) international conference 2014. Current efforts, challenges and future directions for long-term repeated dose toxicity assessment were discussed. Plenary lectures were provided by Richard Judson (US EPA), with a focus on the related US initiatives Tox21 and ToxCast, and Mathieu Vinken (Vice-President ESTIV) who presented the development of the hepatic adverse outcome pathways in the **SEURAT-1** Research Initiative.

4.10.2 Cluster Meeting of the SEURAT-1 Research Initiative

Elisabet Berggren

The **SEURAT-1** Research Initiative assembled for its fourth Annual Meeting in Barcelona, Spain, on 5-6 February 2014. This annual meeting was the largest so far with more than 150 registered participants.

The **SEURAT-1** research projects are now well advanced in their work programmes and a large variety of methodologies and tools were presented at the meeting. These included advanced flow-through 3D bioreactors closely imaging human liver tissue; biomarker discovery including both '-omics' and functional readouts; and differentiation of human pluripotent stem cells (hPSC), resulting in 18 banked and characterised hPSCs ready to be used in assay development. One of the main achievements presented was the release of the open access COSMOS database, which contains structural and toxicity data for more than 80,000 substances including cosmetics ingredients. This database is also available for immediate use by non-**SEURAT-1** partners. ToxBank reported good progress in collecting data for **SEURAT-1** standard reference compounds ('gold compounds') from **SEURAT-1** projects as well as from publicly available sources.

The success of **SEURAT-1** will ultimately be demonstrated through the proofs-of-concept (PoCs) on three levels: The first PoC formulated theoretical descriptions of adverse outcome pathways (AOPs) for three major liver toxic modes-of-action; this was already demonstrated and the results were contributed to the OECD AOP constructs initiative. Further pathway elucidations for different organs were discussed at the meeting, as well as proof and refinement of the AOPs that have already been developed. The second PoC, for setting up systems to predict toxicity, was initiated by seven detailed case study proposals including: prediction of certain specific organ toxicities (e.g. fibrosis and steatosis); organ toxicity based on the main AOPs of the organ predicted through read-across of '-omics' data; classifying liver toxic and non-toxic chemicals using a prediction model based on high throughput *in vitro* data; predicting general toxicity from developing chemotypes for mitochondrial toxicity; and through genomics profiling. The third PoC is the application of AOP theory and predictive toxicity systems developed within **SEURAT-1** in safety assessment. Here, cluster partners intend to further investigate two scenarios; a read-across and a fully quantitative risk assessment scenario.

Achieving consistency across case studies, especially regarding chemical selection, was discussed; this would facilitate the comparison of data from different studies. The results of the predictive toxicity case studies are planned to be presented at the next annual meeting. The ultimate goal of the research initiative is to prove that it is possible to conduct safety assessments of chemicals based on data from the methods developed within **SEURAT-1**.

As by now a tradition at the **SEURAT-1** Annual Meetings, three young scientists were presented

with ‘Excellent Poster Awards’. The subjects of the winning contributions this year were related to kinetic modelling of hepatotoxicity, the COSMOS database, and the case study based on the *HeMiBio* liver bioreactor. 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.

4.10.3 Young Scientist Poster Award

In total, 31 posters were presented at the Annual Meeting, covering diverse research activities in the different projects of the **SEURAT-1** Research Initiative. The poster award committee selected the three best posters, and the awardees present their work in the following extended abstracts.

4.10.3.1 Investigation of the Fibrotic Response Induced by Methotrexate and Acetaminophen in the *HeMiBio* Liver Bioreactor – Part I: Development of a 3D Co-culture Model for *in vitro* Toxicity/Fibrosis Testing

Sofia B. Leite, Tiffany Rossens, Adil El Taghdouini, Mustapha Najimi, Christopher Chesne, Leo A. van Grunsven

Introduction

Chronic liver diseases, e.g. alcoholic liver disease, non-alcoholic fatty liver disease and viral hepatitis, lead to liver fibrosis and subsequent cirrhosis of the liver. According to the WHO, liver cirrhosis accounts for 1.8% of all deaths in Europe, causing around 170,000 deaths per year, with a higher prevalence in eastern and western Europe. However, in terms of fibrosis, no pharmacological agent has been approved for routine use in a clinical context.

Hepatic Stellate Cells (HSCs) have been identified as key players in fibrosis. In the quiescent state, HSCs store vitamin A and have a balanced production of extracellular matrix (ECM). Upon (chronic) injury these cells trans-differentiate into myofibroblasts, in the process releasing vitamin A, and increasing proliferation, motility and deposition of ECM. *In vivo,* this activation can be the result of direct activation of the HSCs, but in the majority of the cases the activation results from the cellular interplay between the distinct liver cells that is mostly initiated by hepatocyte injury.

HSCs activate when cultured, partly due to the contact with a rigid surface (resembling the rigidness of the fibrotic tissue), allowing the study of the activation pathways. Conversely, *in vitro* strategies to keep HSCs quiescent for a controlled activation, and for the testing of (anti)

fibrotic compounds, are still scarce. In order to accurately assess the induction of fibrosis environments by a compound (drug, cosmetic ingredient, etc.), it is important to have a culture model that can keep HSCs in their quiescent state without masking their activation cue, but still preserving their capacity to trans-differentiate. Moreover, fibrotic environments can be better mimicked by the presence of other liver cells, especially hepatocytes, which are the main drug-targets and metabolizers in the liver. Several studies had shown that in co-culture both hepatocytes (*Krause et al., 2009*) and HSCs (*Sawitza et al., 2009*) can benefit from the presence of the other cells; however co-culture strategies that show that both cells can keep their functions simultaneously are still lacking.

Approach

The aim of this work is to develop a reliable *in vitro* co-culture setup (based on physiological characteristics) of hepatic inter-communication for testing toxicity and (anti)fibrotic potential of compounds. This will be completed in three phases as shown in *Figure 4.51*.

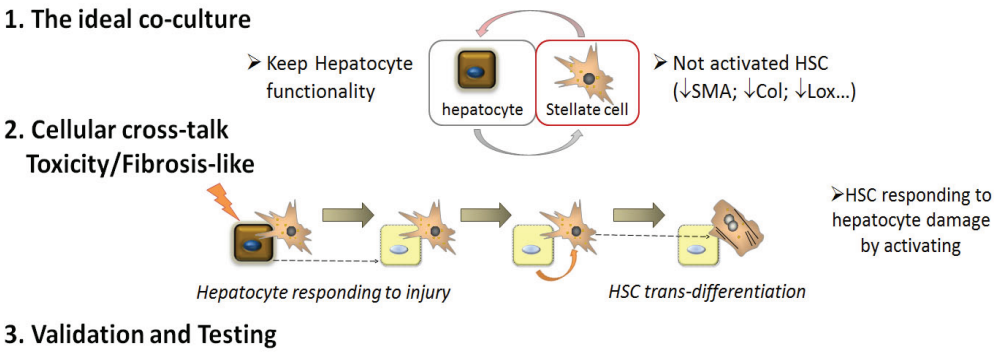

Figure 4.51 *Representative scheme of the hepatocyte-HSC co-culture development for testing pro-fibrotic compounds.*

This work is integrated in the *HeMiBio* project, which is developing a bioreactor micro-device to retain co-cultures of liver cells (hepatocytes, HSC and liver endothelial cells) for drug testing. However, for optimisation of the hepatic cell co-cultures, studies were performed in 96-well plates, keeping the human HSCs and the hepatocytes (here HepaRG cells) in 3D spheroids. For HepaRG monocultures, the spheroid approach was shown to sustain hepatic functions for extended periods of time that are suitable for drug testing (*Leite et al., 2012; Gunness et al., 2013*). After optimisation of 3D co-culture spheroid formation and culture, and using the proper cell ratio and correct media formulation for 3 weeks, the model was tested for the use of a compound that would induce *in vitro* hepatocyte death and subsequent HSC activation.

Acetaminophen (APAP) was used as a reference compound with a known outcome, i.e. hepatotoxicity and perhaps the ability to induce indirectly HSC activation. This also serves as a quality control of the hepatocyte/HSC co-culture since it verifies effective metabolisation by hepatocytes (cells die due to the accumulation of NAPQI, a CYP2E1-mediated metabolite of APAP and this can indicate the good quality of the cells after 21 days) and also the potential of the HSCs to activate upon hepatocyte injury.

Results and Discussion

3D HepaRG/HSC co-cultures were successfully kept for 3 weeks. At the end of the culture time, cells were challenged by the addition of different concentrations of APAP then incubated for 24 hours, after which cell viability was assessed and part of the cells underwent RNA analysis.

The relative viability was calculated based on the ATP levels of cells that were not exposed to APAP. An increased toxic-response (*Figure 4.52*) was observed, allowing the calculation of the EC50 lower than the 3D HepaRG mono-culture. The low value of the EC50 confirms the good metabolic capacity of the hepatocytes – the toxic CYP metabolite (NAPQI) shows to be efficiently produced. Additionally, mRNA levels showed that for the same range of APAP concentrations, there is dose-response increase of fibrotic markers; this was not observed in the control 3D HSC and HepaRG mono-cultures.

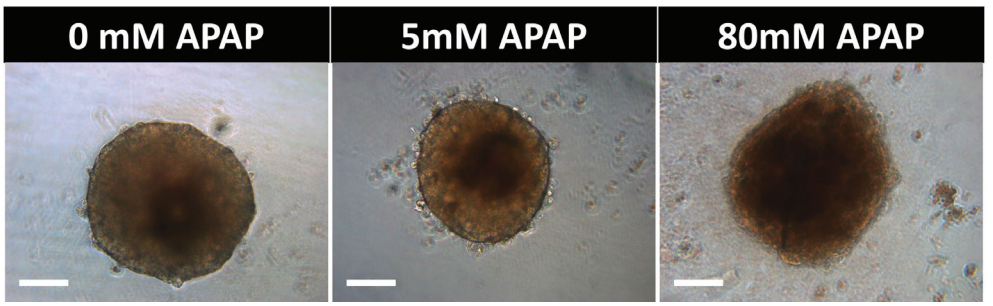

Figure 4.52 *3D HepaRG/HSC co-culture, day 21, after 24h exposure to different concentrations of APAP. White bar represents 100mm.*

Conclusions

These results suggest that 3D HSC-HepaRG co-cultures are a promising *in vitro* model for testing pro- and anti-fibrotic compounds, as well as for general drug-toxicity testing. However, further characterisation of the model, as well as validation with pro-fibrotic compounds such as carbon tetrachloride and methotrexate, should be performed.

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4.10.3.2 TK/TD Modelling to Analyse Real Time Hepatotoxicity Data for Cosmetics

Sophie Teng, Sylvie Barcellini, Roger Rahmani, Remy Beaudouin, Alexandre Péry

Introduction

Recent *in vitro* improvements permit the monitoring of cell responses in real time. With impedance metrics technology it is possible to monitor cell cyto-morphological changes and cell viability over time (*Solly et al., 2004; Cerriotti et al., 2007; Ke et al., 2011*). It then becomes straightforward to assess not only the level of effects but also their dynamics. However, these new tools require a change of paradigm in the way the data are analysed, i.e. moving from descriptive statistics towards the calibration of toxicodynamic models able to account for the temporal variation of the response. In a second step, once this toxicodynamic model is calibrated, its coupling with a physiologically based pharmacokinetic (PBPK) model able to model the temporal variation of hepatic concentration after human exposure will permit the prediction of liver toxicity *in vivo*.

The aim of our work is to propose a methodology of analysing impedancemetrics data by building a mechanistic toxicokinetic/toxicodynamic (TKTD) model to describe *in vitro* cell viability after short and long-term exposure.

Approach

Tests were performed on HepaRG cells with three hepatotoxic cosmetic-related compounds: coumarin, isoeugenol and benzophenone-2. We performed two experiments: one with short-term 48 hour exposure, and one with long-term repeated exposure for four weeks. We built models in R software to analyse the data.

We investigated mathematical models with different hypotheses relative to kinetics: (i) first order kinetics for compound uptake and elimination by the cell or quasi-instantaneous equilibrium between exposure and cell internal concentrations; (ii) decrease of compound concentration due to unspecific mechanisms; and (iii) decrease of compound concentration due to metabolism. To account for toxicodynamics, we used a basic model relating cell population decrease to actual exposure concentration. This had to be improved for benzophenone-2 by adding another differential equation to account for a cell spreading mechanism at an early stage of toxic effects.

We challenged the calibrated models with single and repeated exposure to predict long- and short-term toxicity. Then we coupled coumarin's *in vitro* TKTD model with a previously calibrated coumarin human PBPK model to predict liver toxicity.

Results

For the three study compounds, we first selected the model which was based on a quasi-instantaneous equilibrium between medium and cell concentrations. Using the goodness of fit as the only criteria we were not able to identify the kinetics *in vitro*. Therefore, we used additional experimental and published data to select the most relevant TKTD model (*Figure 4.53*). Indeed, the enzyme responsible for coumarin metabolism, CYP2A6, is less expressed in HepaRGcells than in human primary hepatocytes (*Anthérieu et al., 2002; Aninat et al., 2006; Guillouzo et al., 2007; Jossé et al., 2008; Kanebratt & Andersson, 2008; Hart et al., 2010*). Therefore, for coumarin we chose the model setup with a decrease of compound concentration due to unspecific mechanisms such as evaporation or binding to the plastic. Isoeugenol and benzophenone-2 both have similar phase 2 metabolisms (*Badger et al., 2002; Schlecht et al., 2008; Hong et al., 2013*). Based on the metabolism model, estimated V_{max} values were 1.4 times higher for isoeugenol and 3.7 times higher for benzophenone-2 in HepaRG cells than in human primary hepatocytes. This could be explained by a lower expression of UGTs and SULTs in HepaRG cells as compared with human primary hepatocytes (*Aninat et al., 2006; Jossé et al., 2008; Kanebratt & Andersson, 2008; Hart et al., 2010*). The similar V_{max} ratio between HepaRG and human primary hepatocytes is consistent with the commonly known metabolism pathway of isoeugenol and benzophenone-2. Therefore, the metabolism model was selected for these compounds.

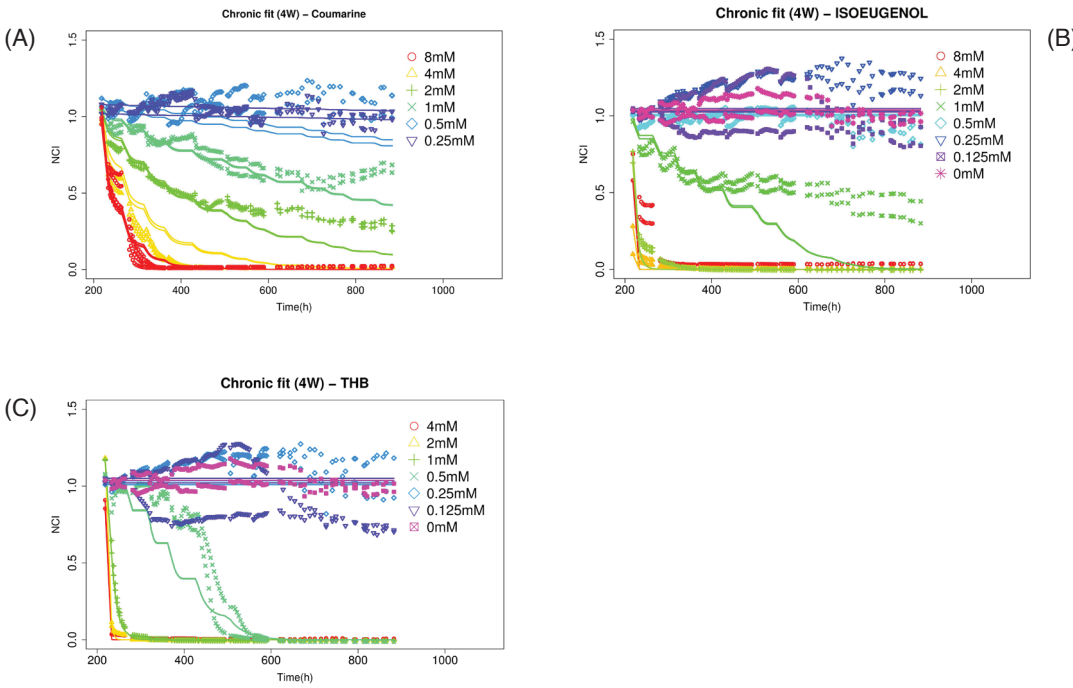

Figure 4.53 *Fits of chronic exposure for four weeks of (A) coumarin (B) isoeugenol and (C) benzophenone-2.*

As shown in *Figure 4.54*, models calibrated on short-term exposure data failed to predict long-term toxicity. In contrast, models calibrated on repeated exposure data could predict short-term toxicity.

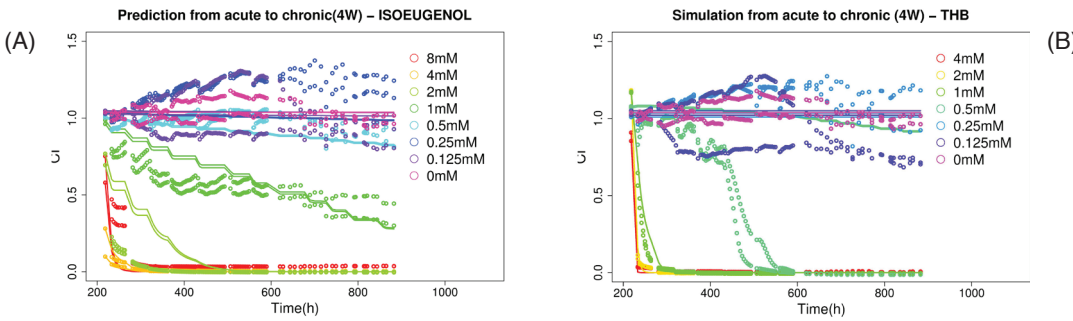

Figure 4.54 *Prediction of repeated exposure for four weeks from parameters estimated by acute toxicity data for (A) isoeugenol and (B) benzophenone-2.*

By coupling our coumarin TD model with the calibrated coumarin PBPK model, we first showed that coumarin is more toxic for slow (ED_{50}=57mg/kg) than fast metabolisers (ED_{50}=88mg/kg; *Figure 4.55*). Then, by simulating a repeated oral administration of the acute no-effect dose (14mg/kg) every eight hours for one week, we highlighted a decrease of hepatocyte viability if the phenotype corresponds to a slow metaboliser, whereas there was no effect on fast metaboliser.

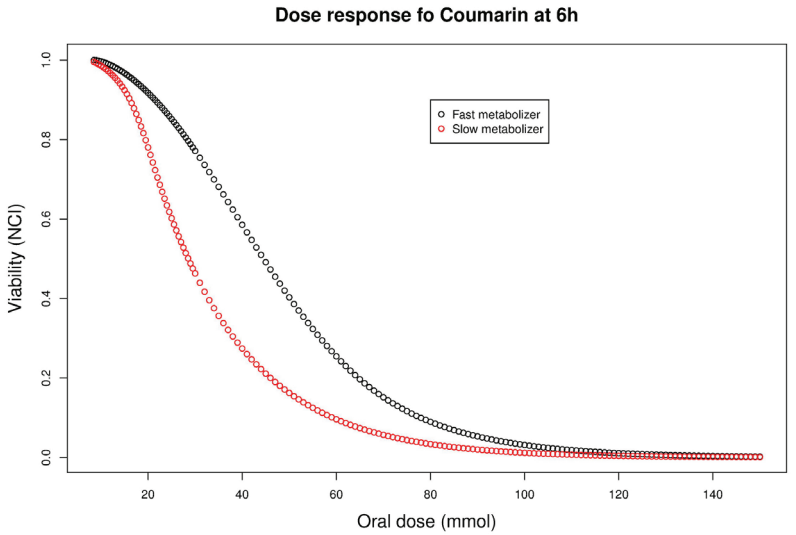

Figure 4.55 *Simulated hepatocytes viability 6 hours after an oral admininstration of coumarin. Red and black circles represent hepatotoxicity profiles of slow (red) and fast (black) metabolisers.*

Conclusions

We proposed a relevant mathematical model to analyse hepatotoxicity data obtained with impedancemetrics and showed that acute data analysis was not fully predictive of chronic data.

Coupling a PBPK model and our TD model for coumarin, we showed and quantified higher sensitivity of poor metabolisers, which was enhanced for repeated exposure.

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4.10.3.3 Collaborative Interoperability between Public Projects to Support Replacement of *in vivo* Repeated Dose Toxicity Testing

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Introduction

COSMOS is one of seven projects forming the **SEURAT-1** Research Initiative and it aims to develop tools and workflows for the prediction of human safety in the use of cosmetic ingredients (see section 4.6). In the framework of COSMOS in particular, and the **SEURAT-1** Research Initiative in general, the management and sharing of chemical and biological data play a central role. To satisfy this goal, the COSMOS consortium developed the COSMOS DB database (http://www.cosmostox.eu/what/COSMOSdb/). The aims of COSMOS DB are: (i) to provide partners and the public with reliable data for repeated-dose toxicity; (ii) to provide partners with tools for data quality assessment; and (iii) to expand the data space through the exchange of content with other international collaborators.

In pursuit of those aims, a web-based system with search capabilities for both chemical structures and toxicity data was developed. The chemistry content of COSMOS DB includes cosmetics inventory from various sources, such as the European Union Inventory of Cosmetic Ingredients (CosIng) database (*European Commission, 2014*) and the US FDA Voluntary Cosmetics Reporting Program; direct food additives and contact substances (FDA PAFA; *Rulis & Hattan, 1985*); and the Distributes Structure Searchable Toxicity Database inventory (DSSTox; http://www.epa.gov/ncct/dsstox/). COSMOS DB also provides data for repeated dose toxicity as well as other important endpoints considered in profiling compounds. COSMOS DB v1.0 has been made publicly available and can be accessed free of charge at http://cosmosdb.cosmostox.eu/ (see also section 4.6.3).

To ensure data consistency and quality, COSMOS DB provides a web-based COSMOS Data Entry System to COSMOS partners. Using this system, the quality of chemical structures and the completeness of toxicity studies are determined at the time of data entry.

COSMOS DB is updated on a regular basis. Scheduled data content updates include the COSMOS Skin Permeability database and the new COSMOS non-cancer Threshold of Toxicological Concerns (TTC) dataset. Through a data exchange program, COSMOS DB will include oral repeated dose studies from the HESS database (NITE Japan; http://www.safe.nite.go.jp/english/kasinn/qsar/hess-e.html).

As a part of the **SEURAT-1** Research Initiative, the interoperability with other systems developed in **SEURAT-1** is very important. One such project is ToxBank – the **SEURAT-1**

cross-cluster infrastructure project (see section 4.8). Interoperability between the COSMOS DB and ToxBank systems within the **SEURAT-1** Research Initiative has been proposed and a simple use case is presented.

Results and Discussion

Chemistry Data: Chemistry data have been collected from various sources. Special emphasis was put on cosmetics-related chemicals. The CosIng and the USA Personal Care Products Council (PCPC) inventories have been included in COSMOS DB. In addition, the US EPA DSSTox (including Tox21 Inventory) and the US FDA CFSAN CERES public content (including **P**riority-based **A**ssessment of **F**ood **A**dditives inventory; *Rulis & Hattan, 1985*) are available.

The public release of the database contains 81,602 chemical records and 44,773 unique (as determined by standard INCHI) chemical structures.

Using the data entry tools provided by COSMOS DB, the COSMOS consortium is undertaking a concerted chemistry quality control (QC) effort. Approximately 450 randomly selected chemical structures have been subject to QC so far. Based on this effort, a quality assessment of the chemical records in COSMOS is as summarised in *Tables 4.14*, *4.15* and *4.16*.

Table 4.14 *Connection tables QA results.*

Records QCed	442
Total Corrected CTs / Error rate	43 / 9.7%
Corrected Connectivity / Error rate	16 / 3.6%
Corrected Stereochemistry / Error rate	24 / 5.4%
Corrected Protonation State / Error rate	3 / 0.7%

Table 4.15 *Chemical names QA results*

Records QCed	442
Total Corrected Names / Error rate	10 / 2.2%

Table 4.16 *Registry numbers QA results.*

Records QCed	442
Total Corrected Registry Numbers / Error rate	2 / 0.45%

Toxicity Data: The public release of COSMOS DB (December 2013) contains 12,538 toxicological studies for 1,660 compounds. Two separate data sets are available: US FDA PAFA and oRepeatToxDB. The US FDA PAFA data set has been generously donated by the Office for Food Additives Safety (OFAS) at the US FDA Center for Food Safety and Applied Nutrition. It contains 12,198 studies across 27 endpoints including: acute toxicity, carcinogenicity, genetic toxicity, neurotoxicity, immunology, repeated-dose, reproductive-developmental studies. The COSMOS consortium harvesting efforts resulted in the assembly of the oRepeatToxDB dataset. Studies from different sources (US NTP and others) have been collected. This has resulted in the collection of 340 *in vivo* repeated dose toxicity studies for 228 chemicals. The oRepeatToxDB study records contain the full plethora of observed toxicological effects together with the corresponding sites at which the effect occurred.

A quality control and assessment of approximately 2% of the records has shown 0.57% erroneous records (e.g., animal counts) and 5.2% missing records (e.g., effect description).

The COSMOS DB quest for new data is ongoing. A number of additional data sets are scheduled for inclusion as a part of the content update cycle. Those include data sets assembled as a part of the project, like the COSMOS Skin Permeability database and the new COSMOS non-cancer TTC data set as well data gathered through a data exchange programme. A successful result of the data exchange programme was an agreement with NITE Japan. This collaboration will allow the inclusion of oral repeated dose studies from the HESS database into COSMOS DB.

COSMOS DB Tools: COSMOS DB provides a rich web-based interface as shown in *Figure 4.56*. This interface runs in all modern internet browsers and allows searching for both chemistry and/or toxicity data. Once the desired data are found they are displayed in a clear and concise chemical compound-centric manner as illustrated in *Figure 4.57*. Complete details about a specific oRepeatToxDB study are available as well as shown in *Figure 4.58*.

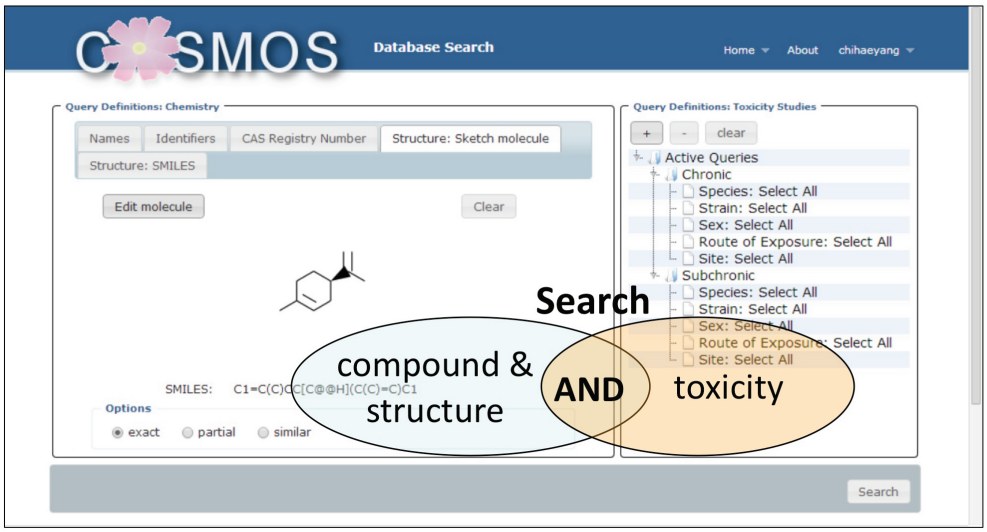


Figure 4.56 *COSMOS DB search interface allows data retrieval by using both chemistry- and/ or toxicity- related queries.*

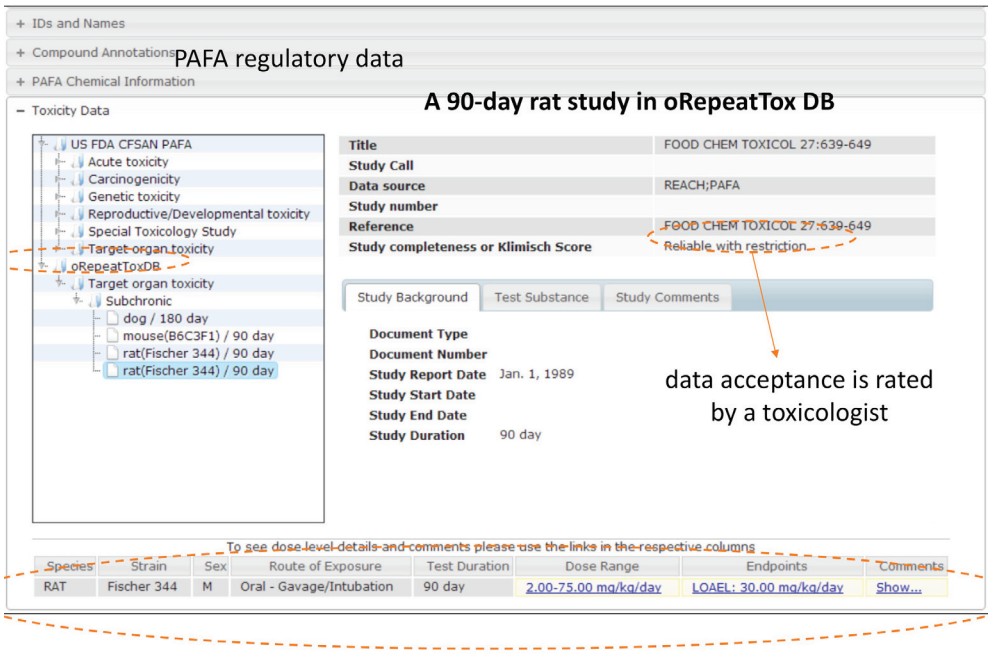
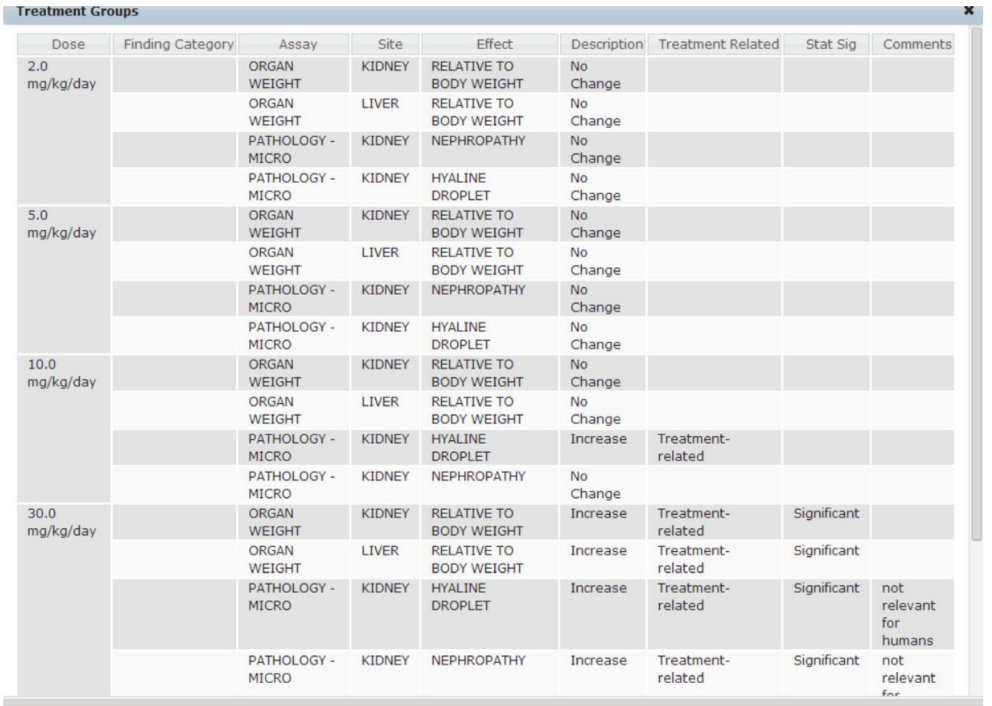


Figure 4.57 *COSMOS DB data display page.*



Treatment Groups

Dose	Finding Category	Assay	Site	Effect	Description	Treatment Related	Stat Sig	Comments
2.0 mg/kg/day		ORGAN WEIGHT	KIDNEY	RELATIVE TO BODY WEIGHT	No Change			
		ORGAN WEIGHT	LIVER	RELATIVE TO BODY WEIGHT	No Change			
		PATHOLOGY - MICRO	KIDNEY	NEPHROPATHY	No Change			
		PATHOLOGY - MICRO	KIDNEY	HYALINE DROPLET	No Change			
5.0 mg/kg/day		ORGAN WEIGHT	KIDNEY	RELATIVE TO BODY WEIGHT	No Change			
		ORGAN WEIGHT	LIVER	RELATIVE TO BODY WEIGHT	No Change			
		PATHOLOGY - MICRO	KIDNEY	NEPHROPATHY	No Change			
		PATHOLOGY - MICRO	KIDNEY	HYALINE DROPLET	No Change			
10.0 mg/kg/day		ORGAN WEIGHT	KIDNEY	RELATIVE TO BODY WEIGHT	No Change			
		ORGAN WEIGHT	LIVER	RELATIVE TO BODY WEIGHT	No Change			
		PATHOLOGY - MICRO	KIDNEY	HYALINE DROPLET	Increase	Treatment-related		
		PATHOLOGY - MICRO	KIDNEY	NEPHROPATHY	No Change			
30.0 mg/kg/day		ORGAN WEIGHT	KIDNEY	RELATIVE TO BODY WEIGHT	Increase	Treatment-related	Significant	
		ORGAN WEIGHT	LIVER	RELATIVE TO BODY WEIGHT	Increase	Treatment-related	Significant	
		PATHOLOGY - MICRO	KIDNEY	HYALINE DROPLET	Increase	Treatment-related	Significant	not relevant for humans
		PATHOLOGY - MICRO	KIDNEY	NEPHROPATHY	Increase	Treatment-related	Significant	not relevant for

Figure 4.58 *COSMOS DB toxicity study results display.*

In addition to data search and retrieval, COSMOS DB provides a data entry system. This system allows the entry of new data as well as the quality control and assessment of old data. When entering new data, the built-in tools automatically check the entry for consistency and common errors. A completeness score using the COSMOS set of criteria is also automatically assigned.

COSMOS DB Interoperability: COSMOS DB provides data integration and links to other related projects within COSMOS in particular and **SEURAT-1** in general. This allows COSMOS DB to better support the needs of the **SEURAT-1** partners. Within the COSMOS project, COSMOS DB has been tightly integrated with COSMOS Space (http://cosmosspace.cosmostox.eu). COSMOS Space is a publicly available resource which facilitates and encourages user interaction and sharing of predictive toxicology resources. COSMOS Space and COSMOS DB share the user base and allow for single sign-on between the two systems. The COSMOS KNIME WebPortal offers tools and computational workflows which are shared across COSMOS partners. Making these workflows available from within the COSMOS DB interface is a work in progress.

COSMOS DB has also established interoperability in the larger framework of the **SEURAT-1** Research Initiative. Within **SEURAT-1**, the ToxBank project established a dedicated web-based warehouse for toxicity data management and modelling, a 'gold standards' compound database and repository of selected test compounds, and a reference resource for cells, cell lines and tissues of relevance for *in vitro* systemic toxicity research carried out across the **SEURAT-1** programme. The project develops infrastructure and service functions to create a sustainable predictive toxicology support resource going beyond the lifetime of the programme. COSMOS DB and ToxBank communicate and exchange data using REST web services compatible with the OpenTox (http://www.opentox.org/dev/apis/api-1.2) specification. Metadata returned by these services are used to provide a seamless integration which links to the original data available across the **SEURAT-1** Research Initiative as illustrated in *Figure 4.59*.

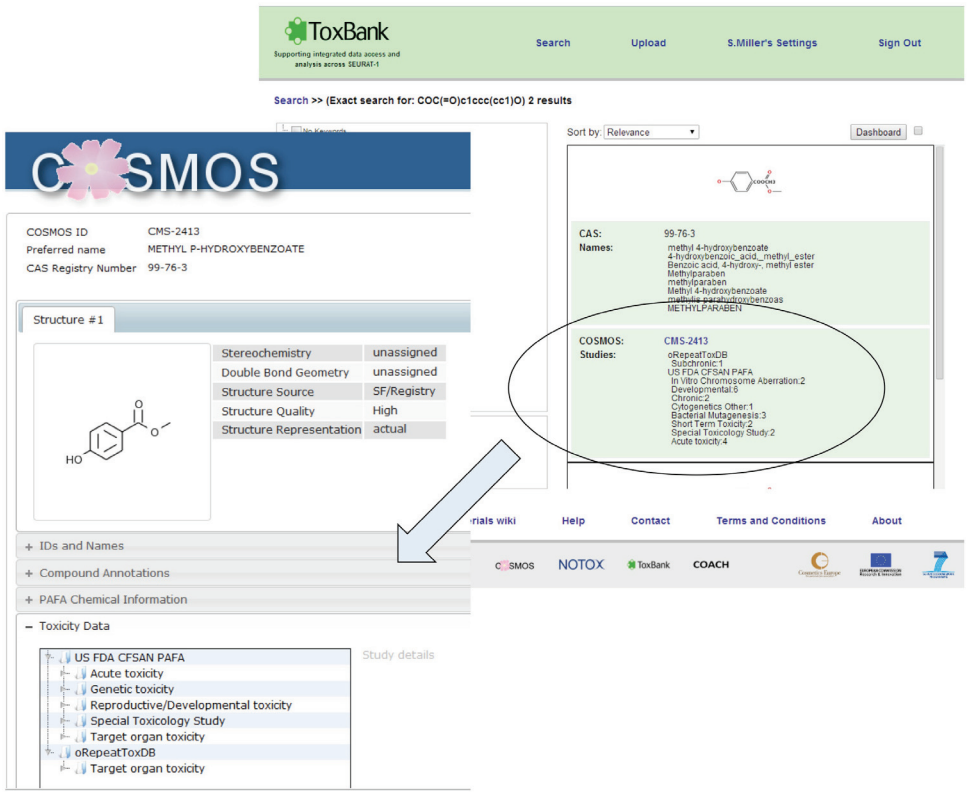


Figure 4.59 *Using COSMOS DB API allows ToxBank to display meta data and to link back to the original COSMOS DB record.*

Conclusions

COSMOS DB – a freely available source of high-quality chemical and toxicological data has been established. In addition to providing data, COSMOS DB provides tools for the project partners which facilitate their day-to-day activities. Interoperability with various related projects both inside COSMOS and within the larger **SEURAT-1** framework has been established thus leveraging the efforts of all partners towards the common goal of replacing animal repeated dose toxicity testing.

References

European Commission (2014): CosIng database, Health and Consumers. http://ec.europa.eu/consumers/cosmetics/cosing/

Rulis, A.M., Hattan, D.G. (1985): FDA's priority-based assessment of food additives: II. General toxicity parameters. *Reg. Tox. Pharm.*, 5: 152-174.

4.11 Cross-Cluster Cooperation

4.11.1 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: the formulation of a mode-of-action-based research strategy; the development of innovative testing methods; the demonstration of proof-of-concept (PoC), thus providing a blueprint for expanding the applicability of the research strategy (objectives are further discussed in section 3.2). **SEURAT-1** projects feed directly into these objectives, either through working groups (see below) or other coordinated cluster activities, and contribute to demonstrating the PoC at multiple levels.

The three levels for PoC studies are intensively discussed in chapter three of this Annual Report. Cross-cluster working groups were established (see the following section 4.10.2) in order to support the design of studies in relation to the three PoC levels. The identified 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 **SEURAT-1** cluster objectives. Altogether, this roadmap will give an overview of cross-cluster interactions and cluster-level milestones, which are formulated to achieve the cluster-level objectives.

The **SEURAT-1** timeline (illustrated in *Figure 4.60*), maps out the milestones of the cluster. It illustrates the timing of PoCs at three conceptual levels and further milestones as the backbone for interactions between the SEURAT-1 projects. In the fifth year, the 'Tools and Methodology catalogue' milestone will comprise 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).

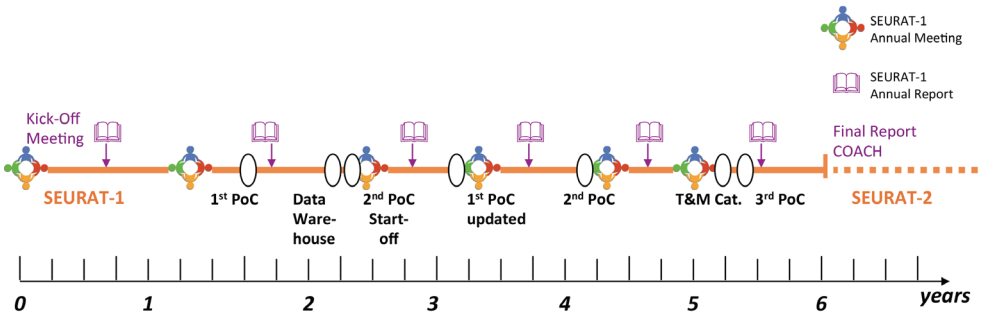


Figure 4.60 *The* ***SEURAT-1*** *timeline.*

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, using the cluster-level objectives to identify and work towards the key deliverables, which are the essential project deliverables for achieving cluster objectives or triggering cross-cluster interactions.

SEURAT-1 project coordinators were first asked to identify the major project milestones, contributing to the **SEURAT-1** objectives (presented in the second **SEURAT-1** Annual Report). They then identified the key deliverables from the project DoW that contribute to these milestones. Based on this, the projects were incorporated into the roadmap and the key deliverables became the basis for the **SEURAT-1** monitoring table and roadmap. 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 segmented maps (not shown). The development of this roadmap was thoroughly discussed in the third Volume of this Annual Report, outlining the contributions from the projects and the working groups to the cluster-level milestones separately. The overall result is summarised in *Figure 4.61* and demonstrates that the **SEURAT-1** Research Initiative continues to progress along its own roadmap. It is anticipated that reporting of level 2 case studies (see chapter 3) will occur at the beginning of 2015, and at the end of 2015 for the level 3 case studies. In addition, a first draft of the **SEURAT-1** Tools and Methods Catalogue will be completed in 2014 based on the key deliverables provided by the **SEURAT-1** projects, which might support the completion of the level 3 safety assessment exercises.

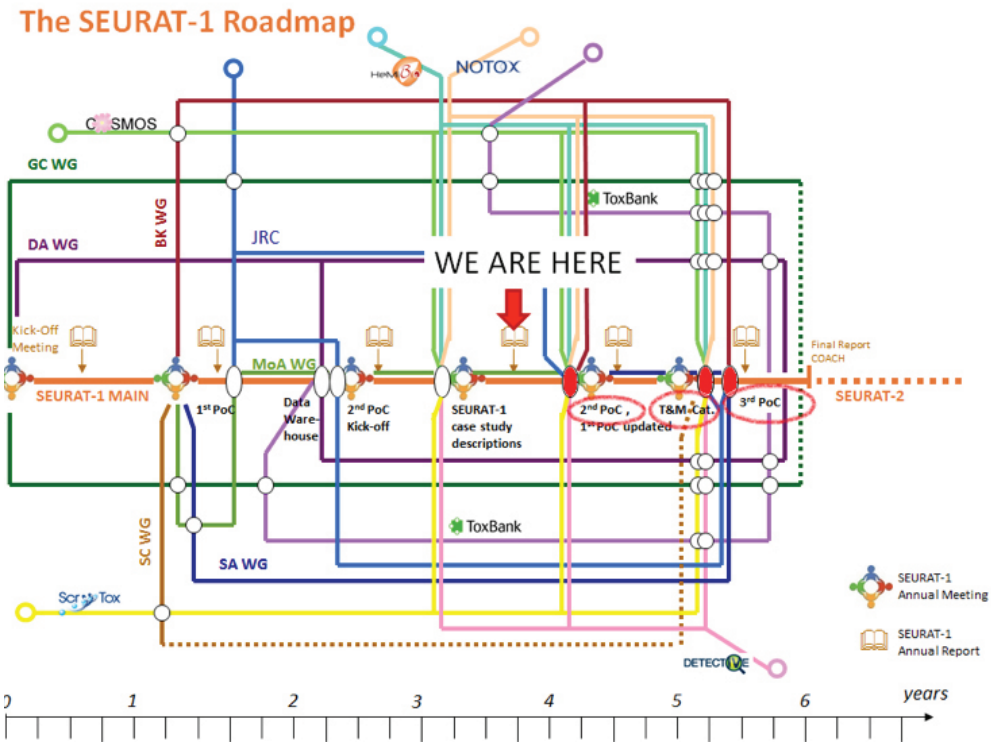


Figure 4.61 *The* ***SEURAT-1*** *roadmap illustrating the contributions from the projects, the JRC and the Working Groups to the cluster-level objectives. GC WG = Gold Compound Working Group, DA WG = Data Analysis Working Group, BK WG = Biokinetics Working Group, MoA WG = Mode-of-Action Working Group, SC WG = Stem Cell Working Group, SA WG = Safety Assessment Working Group.*

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.

4.11.2 The Model of Cross-Cluster Working Groups

The COACH Team

As briefly described in the previous chapter, Working Groups were created to facilitate 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); and

(iii) capture the knowledge spread over more than 70 partners of the **SEURAT-1** Research Initiative. The challenge was to encourage collaborations not foreseen in the individual project deliverables lists and to find a way to broaden the reach of the **SEURAT-1** Research Initiative. It was therefore agreed by the **SEURAT-1** Scientific Expert Panel 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 broad 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 4.17* provides an overview about the **SEURAT-1** Working Groups, including short descriptions (more detailed working group reports are given in the following sections).

***Table 4.17** Overview about the **SEURAT-1** Working Groups.*

Working Group	Co-leaders	WG Description
Gold Compound	Jeffrey 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 modes-of-action.
Data Analysis	Glenn Myatt (ToxBank) Annette Kopp-Schneider (DETECTIVE)	The Data Analysis Working Group holds onging discussions on best practices, standards and common approaches for programme 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 that are required by emerging research activities carried out under the programme. The DAWG contributes also to 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 identified known modes-of-action to support data analysis and outcomes from different projects. The Adverse Outcome Pathway (AOP) framework approach is used 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 Péry (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 assists **SEURAT-1** projects to design *in vitro* and bioreactor models and experiments applied to those. To enable *in vitro* to *in vivo* extrapolation, 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 achieve the **SEURAT-1** objective to develop highly innovative tools and methodology that can ultimately support regulatory safety assessment.
Stem Cells	Glyn Stacey (*SCR&Tox*) Anna Price (DETECTIVE/ *SCR&Tox*)	The aim of the Stem Cells Working Group is to standardise quality control issues of the cells used between different partners and projects. Three cross-consortia cell model subgroups have been 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, makes 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 aims to bridge the gap between non-animal toxicity testing and the safety assessment decision-making needs. Future safety assessment approaches should be based on the comprehensive knowledge of the modes-of-action and pathways leading to adverse effects in humans, rather than on animal testing. The working group focuses 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 examines 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 acts as a facilitator to identify both key gaps in current knowledge and data needs for safety assessment, working across regulatory and science domains to ensure their generation, e.g. they will work with ToxBank to identify negatives that realistically help to define adaptive versus adverse effects.

4.11.3 Gold Compounds Working Group: Mechanism-based Selection of Reference Compounds for the Development of *in vitro* Toxicity Testing Methods

Jeffrey Wiseman, Paul Jennings

4.11.3.1 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*).

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-action*; (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.

4.11.3.2 Compound Summary Table

Phase I of Gold Compound selection is now complete, with compounds selected for hepato-, cardio-, nephro-, and neurotoxicity. Compound selection in this reporting period was focused primarily on these three organs and was influenced heavily by input from the **SEURAT-1** laboratories specialising in these toxicities. We want to especially acknowledge the contributions from these laboratories to the Gold Compound Working Group. The complete list of compounds is available on the ToxBank wiki at http://wiki.toxbank.net/wiki/CompoundSummaryTable and is reproduced below (*Table 4.18*). Detailed descriptions of most of these selected compounds, including extensive tables of properties, may be found on the ToxBank wiki. This collection is termed 'phase I' because it is designed to meet the immediate needs for assay development and validation in the current start-up phase of assay development.

Table 4.18 *Summary information for* ***SEURAT-1*** *standard reference compounds ('Gold Compounds').*

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		
Antimycin A	Mitochondrial disruption, ROS	Cytotoxicity	No
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 Ligands		
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 oxidising 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 inhibitor	Mechanistic standard	No
Nocodazole		Inhibition of neurite outgrowth	No
U0126		Inhibition of neurite outgrowth	No
Acrylamide		Inhibition of neurite outgrowth	No
Propofol		Inhibition of synaptogenesis	No
Lead(II) chloride		Inhibition of synaptogenesis	No
Chlorpyrifos	Organophosphate acetylcholinesterase inhibitor	Affecting cAMP signalling (CREB)	No
Diazinon	Organophosphate acetylcholinesterase inhibitor	Affecting cAMP signalling (CREB)	No
Dieldrin		Affecting cAMP signalling (CREB)	No
Ni^{2+}		Affecting cAMP signalling (CREB)	No
Tributyltin (TBT)		Affecting cAMP signalling (CREB)	No
Trimethyltin (TMT)		Affecting cAMP signalling (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

4.11.3.3 Planned Future Activities

As we approach the end of phase I and move to data generation rather than assay validation, this list will need to be expanded to meet the increased throughput in the laboratories. ToxBank is currently laying the foundation of this expansion by developing a scope and outline for the effort required. A goal in this next phase will be to mine information from large-scale screening efforts (ToxCast being the supreme example) and public data warehouses. The mined data will serve to inform compound selection and also be integrated with data in the ToxBank databases to provide a warehouse of assembled background information for the compound collection.

The Gold Compound Working Group efforts are being coordinated with the Mode-of-Action and Safety Assessment Working Groups, which includes participation in the kick-off meetings for these teams in the fall of 2012 and on-going participation in deliberations in these areas. This cross-working group collaboration is serving to inform expansion of the Gold Compound concept into phase II of compound selection.

The rationale and results behind the selection of hepatotoxins is being written up for publication and the draft manuscript accompanies this report.

References

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4.11.4 Data Analysis Working Group: Integrated Data Analysis

Barry Hardy, Annette Kopp-Schneider, Glenn J. Myatt

4.11.4.1 Introduction and Objectives

The objective of the Data Analysis Working Group (DAWG) is to support the data analysis needs of the **SEURAT-1** Research Initiative, including data collection, integration, analysis, as well as experimental design. It provides a forum to discuss issues or problems within **SEURAT-1** 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 Harmonisation of Processed Data

The ISA-Tab format is currently being used as the data exchange format for investigations across the **SEURAT-1** Research Initiative (*Sansone et al., 2012*). This format defines the experimental design and provides links to the raw and processed data, along with links to protocols describing each step. To support an integrated view of the **processed** data generated from experiments across the projects of the **SEURAT-1** Research Initiative as well as externally, a harmonised file format for this data has been developed. Each type of experiment (e.g. transcriptomics, proteomics, etc.) will have a different file format. The file containing this processed data is to be uploaded as part of an ISA-Tab archive and will be used to enable an integrated analysis of the data over all projects of the **SEURAT-1** Research Initiative as well as integration with other analysis or pathways tools (*Figure 4.62*). This data will also be used in the ToxBank Data Warehouse to support precise searching (e.g. identify all investigations containing a specific gene identified to be statistically significantly under- or over-expressed).

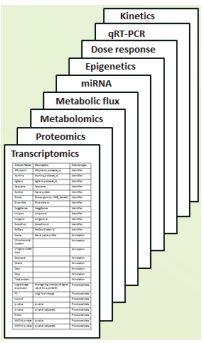

Figure 4.62 *Harmonised file format for processed data as a basis for integrated data analysis.*

The files contain the processed data tables and are formatted differently depending on the type of assay technology used (e.g. transcriptomics, proteomics, etc.), that is, there will be different columns of data for each technology. The data will have information to (i) uniquely identify the genes, proteins, metabolites, etc. (e.g. an Entrez identifier); (ii) annotate the result (e.g. with the name of the gene); (iii) describe the processed results (e.g. fold change comparing genes expressed in the treated sample to the control). It is not necessary to include all data columns, but a minimum set is required. There is flexibility to add additional columns. To illustrate the use of this processed data format, a file was generated from the *in vitro* data of the publicly available TG-GATEs database (http://toxico.nibio.go.jp) for doxorubicin. This formatted file was used directly to perform a KEGG pathway enrichment using the InCroMAP software (http://www.ra.cs.uni-tuebingen.de/software/InCroMAP/). This approach is visualised in *Figure 4.63* with the fold change values color-coded on each gene in the pathway.

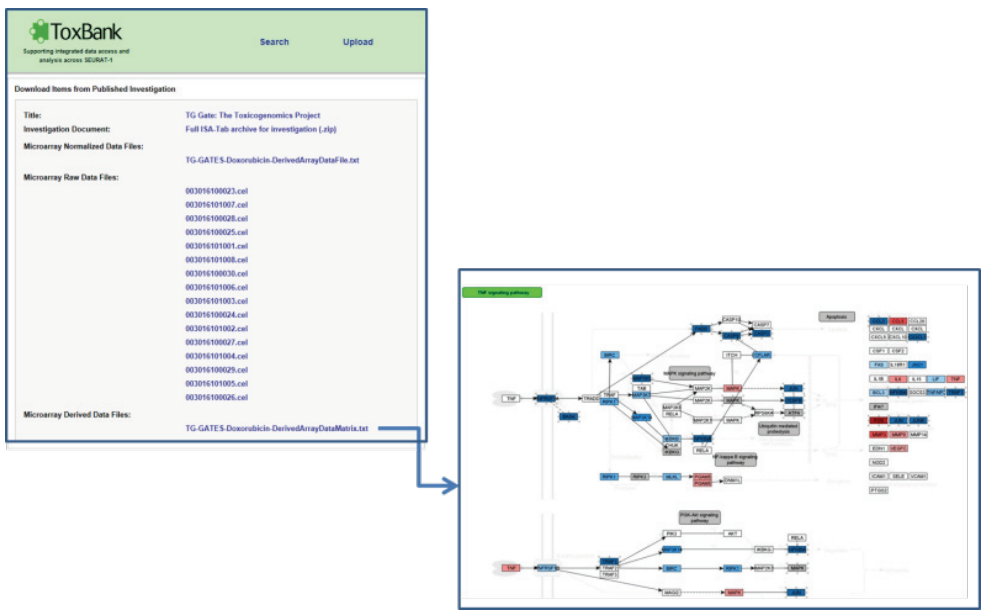

Figure 4.63 *Example of an individual enriched pathway*

4.11.4.3 Online Seminars

To advance data analysis-related issues, a number of DAWG activities and webinars were initiated, including webinars from Wageningen University and the University of Tuebingen, discussing practical approaches to integrated analysis and pathway-centered visualisation of cross-omics datasets and *in vitro* to *in vivo* extrapolations.

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4.11.5 Mode-of-Action Working Group: Capturing Mode-of-Action Knowledge

Brigitte Landesmann, Mathieu Vinken

4.11.5.1 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.

4.11.5.2 Overview of Activities

The MAWG, co-chaired by Brigitte Landesmann (JRC-Italy) and Mathieu Vinken (VUB-Belgium) pursued its activities with particular focus on the implementation of the adverse outcome pathway (AOP) concept into the **SEURAT-1** Research Initiative.

Following the generation of AOP constructs from protein alkylation to liver fibrosis and from liver X receptor activation to liver steatosis, a third hepatic AOP from the inhibition of the bile salt export pump to cholestasis was developed by the MAWG. Assessment of the newly postulated AOP was done by meeting the Bradford-Hill criteria and by answering the OECD key questions. This AOP was published in *Toxicological Sciences* (*Vinken et al., 2013*) and was accepted shortly thereafter into the OECD AOP development programme. Furthermore,

this AOP has been presented orally and by poster at several international meetings and conferences.

As an extension of the AOP development work, a review paper has been published in *Toxicology* (*Vinken, 2013*). This paper gave an in-depth overview of the general strategy to generate and evaluate AOPs. It also exemplified the actual applications of AOPs in toxicology and risk assessment, and proposed some routes for future research and improvement of this field.

In 2013, the different **SEURAT-1** projects have been asked to propose proof-of-concept case studies. The MAWG, by participation of one of its co-chairs in the DETECTIVE and HeMiBio projects, has actively contributed to the development of two cases studies. In fact, these case studies are fully focused on the verification of the established AOPs from protein alkylation to liver fibrosis (*HeMiBio*) and from inhibition of the bile salt export pump to cholestasis (DETECTIVE). Furthermore, an overview paper has been published on the process of selecting modes of hepatotoxic action relevant for cosmetic ingredients, selection of the latter and contribution of the **SEURAT-1** proof-of-concept case studies to the verification and refinement of the three hepatic AOPs introduced by the MAWG (see section 4.2).

In June 2013, the MAWG was represented at the 'SEURAT-1 meets Tox21 workshop' held in Ispra, Italy. The purpose of this event was to seek for opportunities for EU-US collaboration, including research on the modes of toxicological action of chemicals (see section 5.3.1). The MAWG prepared an action plan laying down proposals for a series of joint activities with respect to AOP development and practical verification, thereby relying on the wealth of mechanistic data regarding the toxicity of a diversity of chemicals generated in the Tox21 programme.

In August 2013, and officially kicked off in September 2013, collaboration of the MAWG with the **SEURAT-1** Data Analysis Working Group was established. This collaboration will strengthen the **SEURAT-1** network and will further facilitate communication between the different projects.

Also in 2013, an elaborated AOP from PPARγ ligand-dependent dysregulation to nonalcoholic fatty liver disease was developed and published by MAWG/COSMOS collaborators (*Al Aharif et al., 2014; Tsakovska et al., 2014*). This AOP will be submitted for consideration of inclusion in the OECD AOP project in 2014.

During the **SEURAT-1** Annual Meeting in February 2014 in Barcelona, Spain, a joint session was organised together with the Gold Compound Working Group (GCWG). In this MAWG-GCWG event, different speakers from the **SEURAT-1** projects were asked to give a concise overview of past, on-going and future MAWG-related activities with respect to organ-specific (*i.e.* heart, liver, nervous system, kidney, skin) as well as cross-organ (*i.e.* mitochondrial) toxicity. Not only collaboration with the GCWG was discussed, but also strategies to link MAWG activities to different **SEURAT-1** projects.

4.11.5.3 Planned Future Activities

Among the planned activities for the next year are:

- Active involvement in the practical performance and evaluation of proof-of-concept case studies in different **SEURAT-1** projects;
- Dissemination and communication of MAWG activities at international conferences, including the presentation of developed AOPs;
- Generation of new relevant AOPs and publication in peer-reviewed journals;
- Setting up collaboration with the US Tox21 programme with respect to AOP development and verification;
- Further contribution to the development of an AOP knowledge base in collaboration with JRC, OECD and the US EPA.

Among the planned activities for the following years are:

- Continuous refinement of the established AOPs;
- Project assistance in assay, biomarker and *in vitro* model development with respect to AOPs;
- Looking for opportunities to continue the AOP efforts after completion of the **SEURAT-1** programme.

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4.11.6 Biokinetics Working Group: Quantitative *in vitro – in vivo* Extrapolation

Alexandre R.R. Péry

4.11.6.1 Introduction

There is strong synergy between experimentalists and modellers in projects of the **SEURAT-1** Research Initiative concerning *in vitro* experimentation and the eventual need to perform a proper *in vitro-in vivo* extrapolation. For the *in vitro* systems, modelling can address which system parameters should be modified to obtain a better accuracy of the results. It is also the only way to obtain quantitative and extrapolable results from *in vitro* tests. Modelling of *in vitro* systems requires information, in particular free compound concentrations outside and inside the cells, i.e. *in vitro* partition coefficients. The same applies to the *in vivo* kinetics assessment. In the context of the **SEURAT-1** Research Initiative, toxicokinetic models can be used to help the design of *in vitro* tests by predicting the expected range of concentrations at the target levels for applied doses. Such predictions, based on mathematical models, can easily account for parameter uncertainty, due for instance, to the use of different cell lines or variability in some measurements. As for metabolism, this is still a key issue and the major source of discrepancies between predicted and actual toxicokinetics.

4.11.6.2 Workshop Report from the Biokinetics Working Group Meeting

A Biokinetics Working Group meeting was held between 24–25 September 2013, hosted by the French National Institute for Industrial Environment and Risks in Paris, France. It gathered mathematical modellers (from the COSMOS project) and participants from other **SEURAT-1** projects developing *in vitro* systems (DETECTIVE and *HeMiBio*) as well as from ToxBank and COACH. Furthermore, delegates from Cosmetics Europe attended the meeting.

Mathematical Models for *in vitro* Systems

The first morning was dedicated to presentations of achievements and discussions related to biokinetics and effects modelling of *in vitro* systems. *Alicia Paini* (from the European Commission's Joint Research Centre (JRC) in Ispra, Italy) presented the 'cell-based assays model' developed by the JRC. This model is subdivided in three submodels: the 'fate model' calculates the partitioning of the substance as a function of time in the system, between cells, walls, air (through evaporation) and the formation of metabolites; the 'cell growth model' calculates the cell population dynamics, depending on the cell type; the 'toxicity model' relates cell concentration to cell death rate. These three submodels are inter-dependent. Intended to

be an open access tool, the 'cell-based assays model' has been coded in R and implemented in the KNIME workflow. A video presentation on how the software operates in KNIME was presented.

Céline Brochot (French National Institute for Industrial Environment and Risks, INERIS) focused her presentation on the calibration of PBPK models based on data obtained *in vitro* and analysed through mechanistic models. The *in vitro* systems considered were static or dynamic systems, which could even include two cell types (HepaRG and Caco2 in the illustration presented). To analyse these data with relevant *in vitro* mathematical models, concentration measurements within and also without cells (thus with no metabolism and binding of the substance to the cell) is very useful. In the same way, for complex systems comparing static and dynamic use can be very profitable for the interpretation of the results.

SEURAT-1 *in vitro* Systems

In the afternoon, *in vitro* systems and potential issues that were or could be addressed through biokinetic models were presented. *Leo Van Grunsven* (Vrije Universiteit Brussel, Brussels, Belgium) presented the development of the *HeMiBio* bioreactors. They aim to provide liver *in vitro* tests than can last for a month and account for the variability among cell types (hepatic stellate cells, hepatocytes, hepatic sinusoidal endothelial cells). Bioreactors can be flow-over (one way in, one way out for the fluid generating contact between fluid and cells) or flow-through (one way in, one or two ways out, with flow through the cell culture). Sensors can be on chip (oxygen measurement), off chip (lactate, glucose, glutamate, pH) or in cell (NF-κB, Nrf2). Important characteristics for biokinetics modelling are the fact that the cells are in aggregates, that the developers moved from PDMS to plastics with lower binding and evaporation, and that the system has a microfluidic nature working with small volumes (in the *µ*l-range).

Paul Jennings (University of Innsbruck) presented work to assess early cellular responses which can lead to altered phenotype. The focus was on the kidney, an organ which sees high concentrations of compounds (parent and metabolites), and which shows metabolism and transport. A case study with cyclosporine A was shown. Human renal epithelial cells were exposed and transcriptomics, proteomics and metabolomics were performed. Supernatant and cell concentration measurements of cyclosporine A were used to fit a biokinetics model. This model showed a non-linear relationship between intracellular and exposure concentrations (a factor of around nine for nominal exposure concentrations within a factor of three), supporting the observation of no effect for a nominal concentration of 5mM and activation of the Nrf2-pathway and the unfolded protein-response pathways for a nominal concentration of 15mM.

Robim Rodrigues (Vrije Universiteit Brussel, Brussels, Belgium) presented hepatocyte-like cells derived from human skin stem cells. These cells express enzymatic activities comparable to or even higher than primary human hepatocytes. Biokinetics (related to transport and

metabolism) or toxicodynamic (related to glutathione depletion) measurements could support explanations for differences of sensitivity to acetaminophen between this new cell line and primary human hepatocytes.

Physiologically Based Pharmakokinetic (PBPK) model for *in vitro* to *in vivo* Extrapolation

The second day was dedicated to PBPK models to support *in vitro* to *in vivo* extrapolation. These models can be partially calibrated through QSAR models (for absorption rate, partition coefficients) and *in vitro* data (protein binding, metabolism, absorption through oral and dermal routes). *Alexandre Péry* (French National Institute for Industrial Environment and Risks, INERIS) presented a case study with acetaminophen in which PBPK models were calibrated based on *in vitro* data and *in silico* models, resulting in kinetics predictions close to the ones obtained with a model calibrated based on human data. To predict dose response, the PBPK model was coupled with a toxicodynamic model describing cell viability as a function of exposure concentration and time. This coupling provides more straightforward access to relating exposure scenarios and effects compared to using either the maximal concentration or the area under the concentration curve to try to relate *in vitro* and *in vivo* effects. New experimental methods which follow cell viability as a function of time would be very suitable to support these toxicodynamic models. The predictions of the dose-response for acetaminophen were close to what we know of the acute toxicity of this compound. Refining the model by introducing a 2D-liver allows for liver zonation, taking into account spatial variations in the metabolization rates and, thus, potentially predicts the consequences of repeated dose exposure more accurately.

A simple PBPK model has been coded in R by *Cleo Tebby* (INERIS) and *Alexandre Péry* and was provided to the workshop participants. This model predicts the time-course of the substance concentration in different organs for a given exposure scenario (unique dose or repeated doses). It can combine three routes of exposure (dermal, oral and inhalation). At the moment, the focus is on the liver but other organs of interest such as the heart and kidney can easily be included.

The availability of human PBPK models for the hepatotoxic Gold Compounds was assessed. Models are available for acetaminophen, CCl_4, methotrexate and fluoxetine. Additional models could be proposed for amiodarone, bosentan, chlorpromazine, and valproic acid.

Discussions and Recommendations

The discussions highlighted many interesting points and are summarised as follows:

(i) Co-development of *in vitro* systems and mathematical models:

➠ The cell-based assays model only runs if the biology of the cell is characterised and the substance is known and unique (no mixture);

➠ Mathematical models can account for transport mechanisms but internal concentration measurements need to be provided;

➠ The behavior of aggregated cells can also be modelled but relevant equations and measurements to estimate the parameters (e.g., surface area) of these equations must be considered;

➠ Actual exposure concentrations in the medium (free concentrations) are a minimum requirement for data analysis. Measuring concentrations in the cell is required to support the assessment of effect or no effect. For the compounds of interest, we should assess if there is an analytical method available and, if so, its limit of detection and its ease of use.

Take-home message: *A priori*, mathematical models can be developed to support the analysis of *in vitro* data, using concentration data. Close collaboration between modellers, *in vitro* toxicologists and specialists in chemical analysis is required to target the most appropriate read-out in the system (mechanisms-based read-outs instead of cell viability only, for instance) and to ensure that chemical measurements would permit the estimation of the model parameters.

(ii) Relevant parameters for PBPK modelling:

➠ PBPK models, especially for humans, are not validated (or only partially) for blood concentrations. For example, the human PBPK model developed for formaldehyde is considered to be a very robust model, and yet data are not available for human validation. Increased confidence in the model could be supported by sensitivity analysis, mechanistic information, and data for structural neighbors;

➠ In terms of the prediction of kinetics parameters based on *in vitro* outcomes, data are needed to assess the relevance of the prediction and the need for correction factors (for instance when predicting *in vivo* metabolization rates from *in vitro* cell metabolization rates).

Take-home message: PBPK models are suitable tools to relate *in vitro* exposure to realistic scenarios of exposure. The generic model coded in R, together with necessary parameters, should permit experimentalists to assess the relevance of the tested concentrations with respect to actual exposures. In return, their *in vitro* results will be extrapolated to relevant exposure situations. This extrapolation will include uncertainty assessments, which provide the possibility to reduce uncertainty through more relevant estimates of the PBPK model (*in vitro* partition coefficients, instead of QSAR predictions, or predictions based on cosmetics data only).

(iii) Relevance of the *in vitro* and mathematical models for cosmetics:

- The liver and kidney are generally not expected to be impacted by cosmetics in realistic exposure scenarios, compared to active compounds such as pharmaceuticals, which have a narrow window between therapeutic interest and toxicity. In this way, the use of alternative methods with larger extrapolation factors than when using *in vivo* tests could be suitable for regulatory acceptance;
- The kinetics tools developed for the liver can be extended to the toxicity of other organs. Moreover, models such as PBPK models can be used for route-to-route extrapolation (oral to dermal, or inhalation to dermal), targeting the most relevant route for cosmetics exposure. They are also essential for repeated dose predictions;
- To improve the relevance of the developed *in vitro* models, it would be better to dispose of some 'active' cosmetics compounds which have been abandoned during their development because of liver toxicity;
- Data on cosmetic compounds ($logK_{ow}$ values, other partition coefficients, permeability coefficients) would be suitable to support the development of relevant PBPK models for cosmetics.

Take-home message: The *in vitro* and modelling tools developed within the **SEURAT-1** Research Initiative target the regulatory needs for chemicals with a focus on cosmetics (oral to dermal extrapolation; integration of *in vitro* and mathematical models for predictions at human level; and mechanistic assessment to support regulatory acceptance). All kinetic and effect data available for cosmetics, which would be provided by Cosmetics Europe, would enhance the relevance of the models.

4.11.6.3 Planned Future Activities

Based on the discussion on the working group meeting reported above, the following activities were planned:

- PBPK models are proposed for valproic acid, ochratoxin A, and bosentan, following interests expressed during the meeting, especially for the NOTOX and DETECTIVE projects;
- Cosmetics data should be provided by Cosmetics Europe to support the development of PBPK and *in vitro* models relevant for cosmetics;
- The development of a proposal of a complete biokinetics case study under the umbrella of the **SEURAT-1** proof-of-concept case studies with chemical measurements, toxicological measurements and modelling.

4.11.7 Stem Cell Working Group

Glyn Stacey, Anna Price

4.11.7.1 Introduction and Objectives

SEURAT-1 has initiated a Stem Cell Working Group to support the development of good stem cell culture practice principles and to 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 reviews 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.

4.11.7.2 Overview of Activities

In the first two years of its operation this group has established a set of quality control templates for standardisation of pluripotent stem cell cultures used commonly in toxicology assays. These templates provide a tool to capture key quality control (QC) parameters. Some of the group members also published a review of key QC parameters for stem cell lines (*Pistollato et al., 2012*). In early 2014 Susanne Bremer left the group and **SEURAT-1,** and the Stem Cell Working Group is now reforming with Dr. Anna Price (JRC) replacing Susanne Bremer, and Dr. Sandra Coecke, also from JRC, joining as a new member. In addition to engaging **SEURAT-1** partners on utility of the QC templates that have been developed, the group has also now proposed to revisit the GCCP guidance (*Coecke et al., 2005*) with respect to stem cell culture and assay development standardisation and publish an updated guidance for stem cell lines. The group will also seek to coordinate best practice on core toxicology assay procedures relating to the preparation, storage and use of test and control compounds, with support from Prof. Emilio Benefati of ToxBank. The latter activity on toxicology procedures will be presented, along with other educational content on the ToxBank Data Warehouse.

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4.11.8 Safety Assessment Working Group

Andrew White, Derek Knight

4.11.8.1 Introduction

The Safety Assessment Working Group (SAWG) bridges the gap between the safety assessment decision-making needs and the innovative predictive systems being developed within the **SEURAT-1** Research Initiative. The aim is to harness the mechanistic outputs of the **SEURAT-1** approach and to support the **SEURAT-1** cluster objectives with an emphasis on how this emerging science can best impact and reshape current risk assessment practice.

By way of introduction, the overarching **SEURAT-1** research strategy is to adopt a toxicological mode-of-action approach 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. The aim is to develop a flexible approach to deliver fit-for-purpose assessment of the toxicological properties of a substance, taking into account its properties and the (regulatory) purpose for the prediction. A flexible 'conceptual framework' has emerged from **SEURAT-1** that can be used as a basis for rational combination of information derived from predictive tools to support a safety assessment process or decision to achieve a stated protection goal in the context of repeated-dose systemic toxicity. This framework is intended to set out a structure to guide assessors in devising a fit-for-purpose (or 'bespoke') Integrated Assessment and Testing Approach (IATA) for the particular circumstances and case. The overall outcome is anticipated to be robust as it is not based on single pieces of evidence, but rather a weight of evidence combined in a biologically-rational manner.

An indicative diagrammatic representation of the latest version of this conceptual framework is shown in *Figure 4.64.* Before beginning the assessment, the degree of confidence needed for the prediction is decided, for example, to replace a standard toxicological study in a regulatory

submission or an industry risk assessment of the substance in a cosmetic product. Within a particular exposure context, the assessor may be able to accept a moderate or low degree of confidence in the prediction if human exposure from the use is well controlled and low. Then the idea is to begin with examining existing knowledge from different lines of evidence. In particular, it is important to consider if this is a 'general chemical' expected to be unselective in interacting with biological targets, or a drug/pesticide designed to be selectively biologically-active. Other evidence could include toxicological studies on the substance; read-across from chemical or biological analogues; QSARs and structural alerts; and expert judgement. There are then two parallel lines of consideration:

- 'General' adverse effects not associated with a particular organ but associated with targets present in many cells, tissues and organs or resulting from general chemical activity (e.g. alkylation);
- 'Organ-based' adverse effects.

Both lines of consideration require a review of toxicokinetics/toxicodynamics. The target organs for the parent substance and metabolites would be the focus of more rigorous assessment, with non-target organs also examined using a simplified assessment. Effects on organs can be assessed by (several) AOPs; with the Molecular Initiating Event (MIE) and Intermediate Events (IEs) within an AOP; incorporating existing knowledge and with new data as a combination of *in vitro* assays ('-omics' data, high-throughput data, etc.); and *in silico* predictions – this forms a battery of assessment tools.

The overall assessment is achieved through the evaluation of this information and evidence, including the assessment of the uncertainty associated with the prediction. It may be necessary to improve the assessment if the result is not fit for purpose.

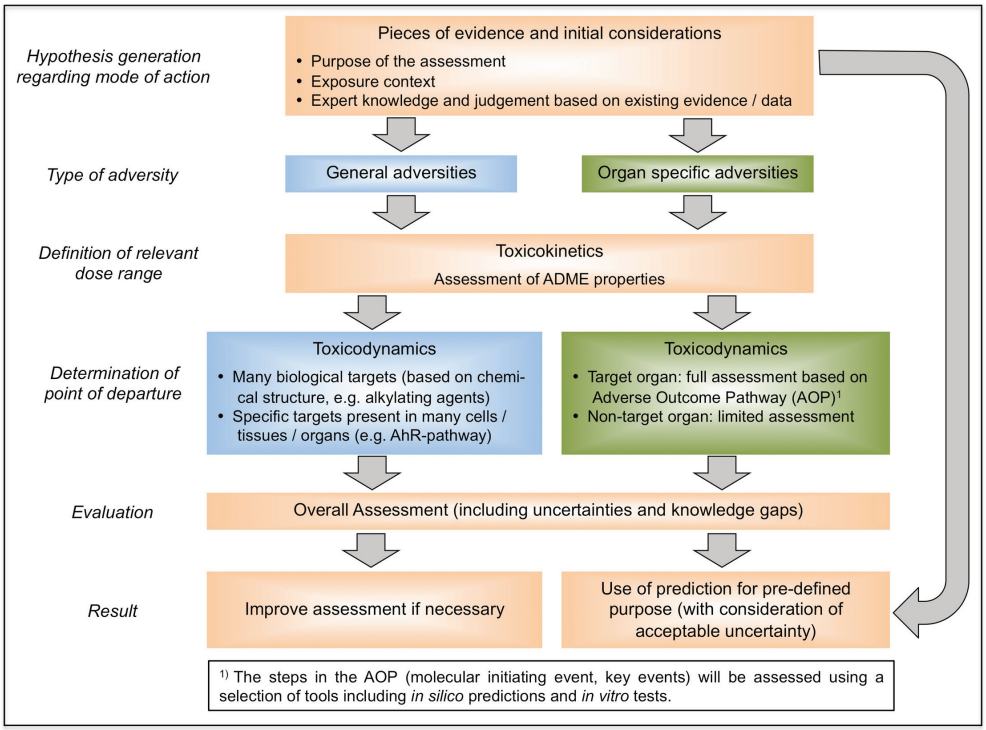


Figure 4.64 *'Conceptual framework' as a structure for assessors in devising a fit-for-purpose 'bespoke' Integrated Assessment Strategy for a particular case.*

Two case studies are being developed in **SEURAT-1** under the lead of the SAWG as key contributions to the proof of concept of the **SEURAT-1** Research Initiative at the application level (i.e. Level 3 case studies, see section 3.5). These two case studies are:

- Improvement of the robustness of the well-established process of assessing the toxicological properties by 'read-across' from a substance of known toxicology to target substance(s).
- An *ab initio* assessment as a 'stretching target' that will highlight gaps for future development and illustrate overall progress made in **SEURAT-1**.

4.11.8.2 Level 3 Case Study on Read-Across Using SEURAT-1 Evidence

The context and progress of this case study is given in section 3.5.1. The aim of the read-across case study is to perform a safety assessment of a substance meeting the standard of regulatory acceptance. Whilst in principle standards can vary between regulators, a good

basis is the standard required for filling a REACH registration information requirement. Conceptually this means that the complete set of results and findings of a 28-/90-day repeated-dose oral rat toxicity study on the 'source' substance should be able to be 'read-across' to the 'target' substance (which has not been studied in animals). The aim is that this prediction is (more or less) equivalent to the omitted standard animal study and it must be adequate for classification and risk assessment (i.e. a DNEL can be set). The application of the 'conceptual framework' will be dependent on the particular pair/category and the known toxicity of the 'source' substance. This was discussed at an expert workshop on 'The read-across case study for safety assessment contributing to the **SEURAT-1** Proof-of-Concept' hosted by the JRC between 29–30 April 2014 (see also section 2.6). Nevertheless, a common key element to examine is the potential for difference in toxicokinetics and toxicodynamics.

An important aspect of the case study is to decide how to examine the added value of the **SEURAT-1** information. This could be by expert judgement of the case before and after the extra evidence is added, to give a qualitative assessment of the robustness of the toxicity prediction. In some cases there may be existing classical animal toxicity data on the 'target' substance to test the 'read-across' predictions against (with and without **SEURAT-1** evidence).

For each of the examples of read-across, it will be necessary to design a suggested testing programme to recommend assays to the **SEURAT-1** community, collect the assay results and integrate them to support the read-across argument, then finally to assess the improvement to the read-across by this extra evidence.

4.11.8.3 Level 3 Case Study *ab initio* Prediction

This case study will show translation of findings and data from integration of relevant Level 2 case studies for a quantitative mechanistic safety assessment. The prediction goal is to determine a safe dose of an ingredient within a consumer use scenario. The approach is described in detail in section 3.5.2 and summarised in *Figure 4.65.* The approach addresses the initial need to determine the critical mode-of-action and then uses higher level integrated models to provide a refined quantitative dose response estimate. These will be compared to published data for Gold Compounds to verify the predictive capacity of the system. Exposures will be modelled based on published biokinetic data for c_{max}, the area under the curve (AUC) and steady state levels; this information will be extrapolated into a dose range for *in vitro* assays.

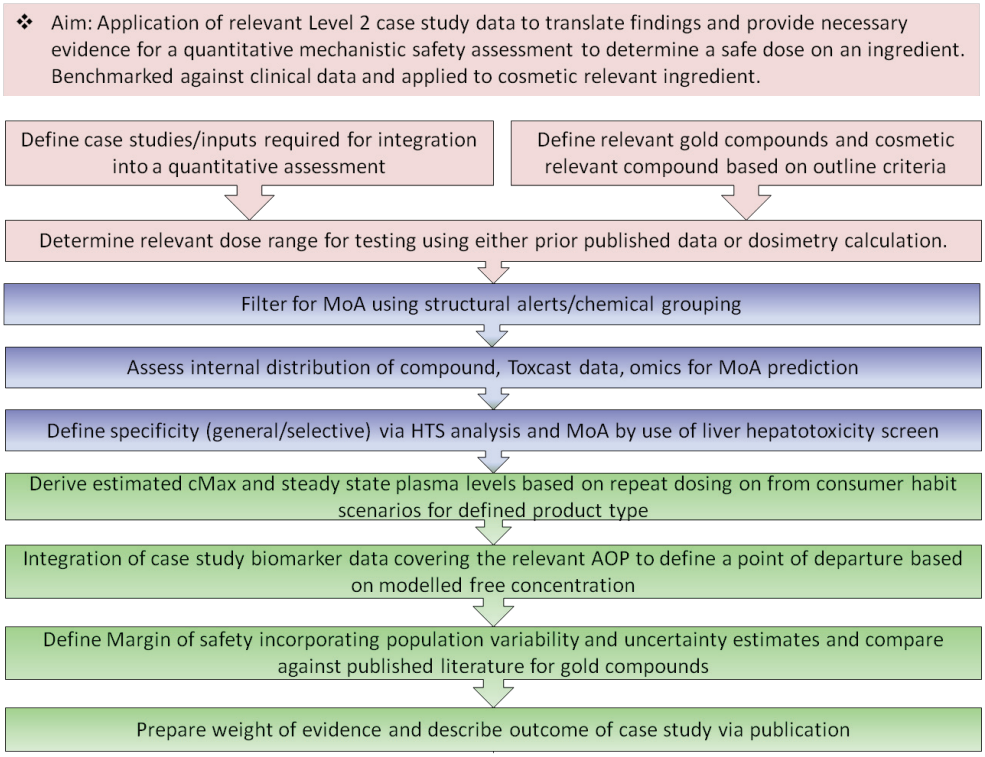


Figure 4.65 *Summary covering main steps in the case study for ab initio prediction leading to an in vitro based quantitative risk assessment.*

Three **SEURAT-1** standard reference compounds (Gold Compounds) were chosen from the list provided by the **SEURAT-1** Gold Compound Working Group (see section 4.11.3) to be used in this case study based on criteria discussed in section 3.5.2: methotrexate, valproic acid and doxorubicin. In addition, a cosmetic-relevant compound will be included. Dose range for testing should cover a range surrounding the predicted required dose, obtained from the lowest human-relevant observed adverse effect for the chosen compound. Doses should be extrapolated to free concentration using PBPK and *in vitro* to *in vivo* extrapolation. For the cosmetic-relevant compound, the *in vitro* doses will be extrapolated from the *in vivo* adverse effect level.

Disclaimer

The views expressed in this paper are solely those of the authors and the content of the paper does not represent an official position of the European Chemicals Agency or Unilever.

4.11.9 Other Workshops

In addition to the Working Group meetings, other workshops were organised to address the 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. Much of the content is confidential and, at this time, cannot be reported here. However, a summary report about a workshop organised by *HeMiBio* highlighting collaborations between different **SEURAT-1** consortia provides some insights into the usefulness of these workshops, and a short note about the first public NOTOX Satellite Meeting finalises this section about cross-cluster collaborations.

The *HeMiBio* Joint Meeting with *SCR&Tox*, NOTOX and DETECTIVE on Bioreactors and Genetic Engineering of Cells

HeMiBio organised a second joint meeting in Leuven, Belgium, on 10 September 2013. As in the first meeting (reported in the third **SEURAT-1** Annual Report), the aim was to connect researchers, facilitate the 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 by Principal Investigators from the different projects who reported on progress with respect to the above-mentioned areas.

As a result of the joint meetings and industry discussions organised during the third **SEURAT-1** Annual Meeting between 6–7 March 2013 in Barcelona, the following specific collaborations have been established: (i) between Catherine Verfaillie, KU Leuven (*HeMiBio*) and Bob van de Water, Universiteit Leiden (DETECTIVE), to incorporate the constructs created in the Leiden laboratory in the RMCE cassette of the iPSC lines generated at KU Leuven; (ii) between Catherine Verfaillie, KU Leuven (*HeMiBio*) and Anders Aspergen of Cellectis AB (*SCR&Tox*) to compare hepatocytes generated from iPSC and perform epigenetic and metabolomics studies in KU Leuven. In addition, the CYP3A4 line generated at KU Leuven will be differentiated by Cellectis AB to assess the suitability for selection of CTP3A4 cells using their optimised differentiation protocol. Finally, (iii) between Leo van Grunsven, Vrije Universiteit Brussel (*HeMiBio*) and Christophe Chesne of Biopredic (NOTOX) for optimisation of HepaRG/HSCs 3D co-cultures.

NOTOX Satellite Meeting

The first NOTOX Satellite Meeting took place on 10 June 2014 in Egmond aan Zee, The

Netherlands. This public meeting was organised in the context of the European Society of Toxicology In Vitro (ESTIV) International conference 2014. The NOTOX Satellite Meeting brought 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 focused on Systems Biology approaches in predictive toxicology using computer models.

4.12 Training and Outreach

Sara Vinklatova, Emmanuelle Da Silva, Bruno Cucinelli

4.12.1 Training Activities

Introduction

Since its beginnings in 2011, training has been one of the important components of **SEURAT-1** outreach activities. A common **SEURAT-1** training strategy has been developed at the initiative of COACH. The COACH team had seen the need for such a cluster level harmonisation initiative due to the fact that the work programme of each of the six individual research projects had been defined independently, and each consortium had defined its own training approach.

In 2011 COACH analysed the training activities of the individual projects and elaborated a proposal for a cluster training concept. In order to be able to coordinate a common training programme, COACH invited the research projects to set up a special task force (**SEURAT-1** Training Task Force – STTF) composed of all seven projects' representatives in charge of the training activities. In 2012, the implementation of the agreed programme started, with the first cluster-level **SEURAT-1** Summer School, an event evaluated as successful by the participants. The STTF further agreed that in 2013 the individual research projects should have the opportunity to implement project-level training programmes, focusing on internal training needs within their project and that there should be no overlap with central **SEURAT-1** training activities. During 2013, COACH took the opportunity to start preparing the organisation of the next cluster-level training activities for 2014. Based on the analysis of the first **SEURAT-1** Summer School feedback made in 2013, COACH identified the main shortcomings and came up with proposals how to address them. *Figure 4.66* illustrates the chronological development of the strategy until today.

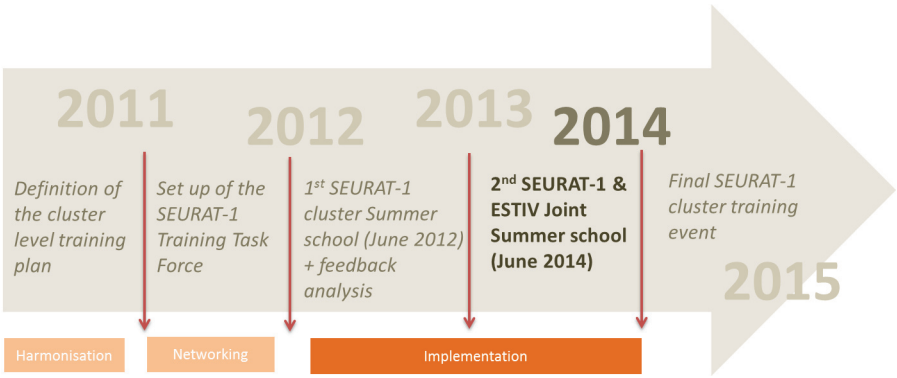


***Figure 4.66 SEURAT-1** training activities.*

SEURAT-1 Summer School 2014

The second **SEURAT-1** cluster level Summer School was organised in collaboration with the European Society of Toxicology In Vitro (ESTIV) – the leading organisation in Europe that strengthens the scientific network of *in vitro* toxicologists and promoting *in vitro* toxicology. This partnership should initiate connections and networking that are important for spreading knowledge.

The **SEURAT-1** & ESTIV Joint Summer School took place from 8–10 June 2014 and was followed by the ESTIV2014 conference. *In vitro* and *in silico* toxicologists from many different countries that represent academia, industry and regulatory bodies were gathered at this event. Given that *in vitro* and *in silico* toxicology are cornerstones of the **SEURAT-1** Research Initiative, it is clear that there is a considerable overlap in the **SEURAT-1** and ESTIV2014 target audiences. The venue of these two events was intentionally chosen to be identical (the Zuiderduin hotel, Egmond aan Zee, the Netherlands), giving Summer School participants the unique opportunity to meet toxicology experts attending the ESTIV2014 conference and also experts from other domains participating in the following two satellite workshops organised in the same hotel: NOTOX Satellite Meeting, focussing on Systems Biology approaches in predictive toxicology using computer models; and the ESTIV-CAAT-IVTIP Pre-congress Workshop, addressing the industrial and regulatory implementation of non-animal integrated testing strategies.

During the organisation of the **SEURAT-1** & ESTIV joint Summer Schools, emphasis is always placed on feedback analysis from the previous year. The 2012 **SEURAT-1** Summer School follow-up questionnaire indeed revealed a couple of points where improvements could be considered for the next event organisation, mainly with respect to the venue, the programme and the session attendance. The identified shortcomings and the actions taken to address them at the 2014Summer School are reflected in *Table 4.19*.

Table 4.19 *Feedback from the* ***SEURAT-1*** *Summer School 2012 and impact on the planning for the* ***SEURAT-1*** *Summer School 2014.*

Summer School 2012 feedback	Summer School 2014
The research project partners should be encouraged to propose more practical sessions. Long theoretical conference-type sessions should be avoided.	The programme is composed of short practical sessions, including: (i) soft skills sessions on scientific writing and science communication; (ii) hands-on computer sessions from NOTOX, COSMOS and ToxBank; (iii) job opportunities session organised during the ESTIV2014 conference giving the opportunity to discuss and broaden the career options for modern toxicologists in an informal setting.
The cluster should increase the visibility of its training activities and appeal for **SEURAT-1** and non-**SEURAT-1** researchers, to ensure higher attendance of training events.	The main goal of the **SEURAT-1** and ESTIV collaboration was to increase the attractiveness of the Summer School. Hundreds of experts participating to the ESTIV2014 conference and the satellite meetings came to Amsterdam, and potential attended the Summer School poster session. Moreover, **SEURAT-1** sponsored one session at the conference and thus gained more visibility.
To organise the next Summer School at a more central location so that the travel costs and time of the participants are reduced.	The Summer school 2014 in organised close to Amsterdam, the Netherlands, one of the most central European locations. Free shuttle buses from the Amsterdam train station to the hotel were organised by COACH.

In addition to the above improvements, COACH planned several activities that were attractive for the participants:

➠ COACH decided to promote the Summer School as much as possible to generate more interest in the scientific press in this important cluster activity. To this end, COACH decided to hire a student film team via the 'Centre for Media Competences', University of Tuebingen, one of the COACH partners. This film team came to the Summer School to create a short video documentary. This video, which will cover the poster session, individual interviews and other inside scenes, will then be used as **SEURAT-1** promotional material at upcoming events, such as the 9th World Congress on Alternatives and Animal Use in the Life Sciences, held in Prague in August 2014.

➠ In order to stimulate the informal exchanges between the participants, many social activities were planned, such as common lunches, one common dinner at the hotel restaurant, a bowling event, drinks at an Irish bar and others. The participants also benefitted from the cosy environment of the Egmond aan Zee seaside resort, where they could rent a bike in the hotel bike store or discover the beach walks.

The registrations for the Summer School were launched in March 2014 via a registration

website prepared by COACH (see *Figure 4.67*). COACH collaborated closely with the ESTIV logistic organisers and developed a common system of registrations, facilitating the inscriptions.

***Figure 4.67 SEURAT-1** & ESTIV joint Summer School registration website.*

The STTF hopes to have organised a fruitful event fulfilling the strategy that was developed over the years 2011–2014. The outcomes and the participants' feedback will be published in the next Annual Report in 2015.

Training Activities

2013 was dedicated to individual project-level training events according to internal needs, in particular hands-on lab training. This was because no cluster-level central training activity was foreseen for this year, as mentioned previously. The following training events took place in 2013 and the beginning of 2014:

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.

HeMiBio Winter School: The Third *HeMiBio* Winter School 'From Stem Cells to Liver Cells",

(also open to non-*HeMiBio* members) was organised by Catherine Verfaillie, Aernout Luttun (KU Leuven) and Leo van Grunsven (Vrije Universiteit Brussels) just before the Annual Meeting on 21–22 January 2014 in Leuven, Belgium.

Indeed, the use of stem cell-derived hepatic cells to populate the different bioreactors that are being constructed is an important aspect of the *HeMiBio* consortium. The Winter School introduced the participants to the biology of (induced pluripotent) stem cells, their generation, epigenetic memory and differentiation potential. The biology of the liver from the lab to the bedside was also addressed, including the function of each *HeMiBio* cell type in health and chronic liver disease or acute liver failure. Practical sessions about the techniques necessary to study stem cell differentiation towards the hepatic cell types included topics like fluorescence-activated cell sorting (FACS), qRT-PCR, fluorescent immunocytochemistry and microscopy. The presentation of a poster was obligatory for all participants and a poster prize was awarded. The Winter School ended with a 'Liver Quiz' which covered the topics of all lectures as well as the practical sessions.

DETECTIVE Summer School: The DETECTIVE Summer School took place in Slano, Croatia, on 10–14 June 2013. It offered a wide variety of subjects combined with interesting activities which made it a real success. Topics included: transcriptomics, epigenetics, functional readouts including high content imaging, *in vitro* toxicity systems, metabolomics, (phospho) proteomics, other keynote lectures and hands-on training. During the 4 days summer school attendees followed oral and poster presentations consisting of practical and theoretical aspects covering DETECTIVE experimental models and methods, adverse outcome pathways and stress responses, overviews on metabolomics techniques, as well as hands-on sessions dealing with '-omics' data management and analysis. Several invited speakers addressed topics related to safety evaluation of cosmetic ingredients, and career opportunities in industry were presented. Additionally, delegates from the eTOX project (http://www.e-tox.net) and the European Medicines Agency were invited and presented overviews about the qualification of biomarkers. The event allowed research partners to get to know each other better and promoted exchange about their scientific work. Improved project partner interaction facilitates the continuous remote work between partners and generates the dynamics that motivates DETECTIVE scientists to push forward the collaborative research work started in the project. The DETECTIVE Summer School was appreciated by all participants for the programme, the interactions and for the venue.

COSMOS Webinar: An open webinar 'COSMOS DB: A New Database of Toxicological Information to Support Knowledge Discovery' was presented on 26 February 2014, hosted by the American Society for Cellular and Computational Toxicology (ASCCT). The available 125 places were fully booked. A recording of the webinar is available from the COSMOS

website along with a short tutorial with examples on how to use the different search options of COSMOS DB (http://www.cosmostox.eu/what/COSMOSdb). Further webinars on COSMOS-related topics are planned.

4.12.2 Workshops

Within **SEURAT-1** a number of workshops were held in the third year and more are currently being organised by the cross-cluster working groups (see chapter 4.11.2–4.11.8). In addition to these, several workshops took place outside of working group activities to address specific aspects of repeated dose systemic toxicity. Respective reports are given in chapter 4.11.9. Furthermore, a **SEURAT-1** workshop entitled 'The Read-Across Case Study for Safety Assessment contributing to the **SEURAT-1** Proof-of-Concept' took place at the Joint Research Centre (JRC) in Ispra, Italy, on 29–30 April 2014. The full workshop report will be published in the next Annual Report. A personal opinion about the importance of this read-across case study and the rationale behind chemical selection in this context is given in section 2.6.

4.12.3 Conferences

SEURAT-1 Stakeholder Event

The third volume of the **SEURAT-1** Annual Report was published in July 2013, distributed in August 2013 and officially launched on 5 September 2013 in Brussels during the **SEURAT-1** Stakeholder Event. This highly appreciated conference, organised by **SEURAT-1** in collaboration with EPAA, aimed to reach out to policy makers, regulators, industry and animal welfare groups to provide information on the progress made so far by the consortium. Around 50 participants attended an afternoon of **SEURAT-1** highlights, achievements and success stories, followed by the official book launch, drinks and a poster session (*Figure 4.68*). The lively discussion that followed the presentations underlined:

- The strong interest of industry in this research work and its continued interest in research and innovation in this field enabling ultimately to deploy new safety assessment solutions in various industrial sectors (cosmetics industry, pharmaceutical industry, chemical industry, medicine, etc.);
- The need to inform and involve the regulators on a much larger scale to make them aware of the changes on the horizon in the field of alternative animal-free human safety assessment methods;
- The willingness of other private and public research initiatives in this field to cooperate and exchange knowledge, avoiding the duplication of effort and supporting faster progress;

- The impact of the revolutionary concepts promoted by the **SEURAT-1** Research Initiative (and others), not only on safety assessment, but also for protection of human health in general, e.g. through the prevention of diseases triggered by environmental factors;
- The need for a global research strategy to identify the next steps of the long-term research and innovation effort.

***Figure 4.68 SEURAT-1** & EPAA Stakeholder Event.*

EPAA joined forces with the **SEURAT-1** Research Initiative to make this event possible and ensured broad dissemination through its extensive member network. As a follow-up to this conference, a press release was prepared and widely circulated through the web from a number of homepages:

- AltTox (http://www.alttox.org)
- Irish Cosmetics, Detergents and Allied Product Associations (http://www.icda.ie/)
- Chemical Watch (http://chemicalwatch.com; registration obligatory)
- The European Commissions' Joint Research Centre (http://ec.europa.eu/dgs/jrc/)
- Cosmetics Europe (https://www.cosmeticseurope.eu)
- **SEURAT-1** website (http://www.seurat-1.eu)

Other Conferences

The **SEURAT-1** Research Initiative was further represented via its cluster projects at a number of international conferences, as summarised in *Table 4.20.*

Table 4.20 *Presence of the* ***SEURAT-1*** *Research Initiative in international conferences and workshops.*

Conference	Date	Place	Contribution	Project
Gordon Research Conference on Mycotoxins and Phycotoxins	16–21 June 2013	Easton, USA	Oral presentation	DETECTIVE
Colloque de l'Association pour la Recherche en Toxicologie (ARET)	20–21 June 2013	Paris, France	Oral and poster presentations	COSMOS
5th International Symposium on Methods and Applications of Computational Chemistry	1–5 July 2013	Kharkiv, Ukraine	Oral and poster presentations	COSMOS
International Conference on Fuzzy Systems (FUZZ), 2013 IEEE	7–10 July 2013	Hyderabad, India	Oral and poster presentations	COSMOS
EPAA Workshop: Stem cell-derived organ-like models for analysing mid- and long-term dosing dynamics	28–29 August 2013	Brussels, Belgium	Poster presentations	
49th Congress of the European Societies of Toxicology (EUROTOX 2013)	1–4 September 2013	Interlaken, Switzerland	Oral and poster presentations	COSMOS ToxBank
18th European Congress on Alternatives to Animal Testing, 15th Annual Congress of EUSAAT	15–18 September 2013	Linz, Austria	Distribution of the 3rd **SEURAT-1** Annual Report, Presentations and posters	COACH ToxBank COSMOS HeMiBio
OpenTox Euro 2013	30 September – 2 October 2013	Mainz, Germany	Oral and poster presentations; co-sponsored by ToxBank	ToxBank *HeMiBio*
CAAT Joint Information Day on "High-Content Imaging Technology in Safety Sciences"	24 October 2013	Mainz, Germany	Presentation	DETECTIVE
18th Brazilian Congress of Toxicology	7–10 October 2013	Porto Alegre, Brazil	Poster and oral presentation	DETECTIVE *HeMiBio*
7th International Symposium on Computational Methods in Toxicology and Pharmacology Integrating Internet Resources (CMTPI-2013)	8–11 October 2013	Seoul, Korea	Oral and poster presentations	COSMOS
Skin Forum's 2nd Skin Metabolism Meeting	10–11 October 2013	Valbonne, France	Oral and poster presentations	COSMOS
International Workshop on Risk Management and Control of Chemicals	13–16 October 2013	Dalian, China	Oral presentations	COSMOS
UK-QSAR and ChemoInformatics Group Meeting	15 October 2013	Alderley Park, England	Oral and poster presentations	COSMOS
HPCI CEE Congress Home and Personal Care Ingredients Central and Eastern Europe	15–16 October 2013	Warsaw, Poland	Distribution of the 3rd **SEURAT-1** Annual Report, Presentation	COACH
CAAT information day on high content imaging chemical safety testing	24 October 2013	Konstanz, Germany	Oral and poster presentations; distribution of the third **SEURAT-1** Annual Report.	COACH DETECTIVE

OpenTox USA 2013	29–30 October 2013	Raleigh-Durham, USA	Oral and poster presentations; co-sponsored by ToxBank	COSMOS ToxBank *HeMiBio*
22th International Federation of Societies of Cosmetic Chemists Conference (IFSCC)	30 October – 1 November 2013	Rio de Janeiro, Brazil	Distribution of the 3rd SEURAT-1 Annual Report, Presentation	COACH
2nd Annual Meeting of the American Society for Cellular and Computational Toxicology (ASCCT)	31 October 2013	Bethesda, MD, USA	Distribution of the 3rd **SEURAT-1** Annual Report, Presentation	COACH
IVTS Annual meeting, *In Vitro* Toxicology Society	4-5 November 2013	University of Leicester, UK	**SEURAT-1** session, oral presentations and distribution of the third **SEURAT-1** Annual Report	COACH COSMOS DETECTIVE *HeMiBio* NOTOX *SCR&Tox,*
International Workshop on Risk Assessment of Cosmetics	7 November 2013	Seoul, Korea	Oral presentations	COSMOS
EPAA annual meeting	13 November 2013	Brussels, Belgium	Distribution of the third **SEURAT-1** Annual Report	COACH
Congrès Annuel de la Société Française de Toxicologie	14–15 November 2013	Paris, France	Oral presentations	COSMOS
International Congress of Pharmaceutical Science 2013 (CIFARP)	20–23 November 2013	Sao Paulo, Brazil	Oral presentations and distribution of the **SEURAT-1** leaflets with the embedded USB sticks	COACH COSMOS
BelTox Annual Meeting	6 December 2013	Louvain-La-Neuve, Belgium	Oral and poster presentations	DETECTIVE COSMOS
9th Annual International Conference on Predictive Human Toxicity and ADME/ Tox Studies	30–31 January 2014	Brussels, Belgium	Poster presentations	NOTOX
Dutch Society of Toxicology Meeting	12 February 2014	Leiden, The Netherlands	Oral presentation	DETECTIVE
7th Annual ADME & Predictive Toxicology	18-19 February 2014	Barcelona, Spain		
3rd Computationally Driven Drug Discovery Meeting (CSSS)	4–6 March 2014	Verona, Italy	Oral and poster presentations	COSMOS
53rd Annual Meeting of the Society of Toxicology (SOT 2014)	23–27 March 2014	Phoenix Arizona, USA	Oral and poster presentations, distribution of the third **SEURAT-1** Annual Report	NOTOX COSMOS ToxBank COACH
49th annual meeting of the European Association for the Study of the Liver (EASL)	9–13 April 2014	London, UK	Poster and oral presentations	*HeMiBio*
UK-QSAR and ChemoInformatics Group Meeting	29 April 2014	Windlesham, England	Oral presentations	COSMOS

9th World Congress, Prague, Czech Republic

An action plan was established to highlight the participation of **SEURAT-1** at the 9th World Congress on Alternatives and Animal Use in the Life Sciences, Prague, August 2014. A variety of promotional activities will be arranged by **SEURAT-1** partners, such as interviews, short scientific sessions, games, videos, and PowerPoint slides. Other events are planned to celebrate the launch of this fourth Annual Report at the **SEURAT-1** corner (part of the JRC booth). The launch is scheduled just after the session on Repeated Dose Toxicity chaired by Michael Schwarz (COACH).

4.12.4 SEURAT-1 Public Website

Since its launch in 2011, the **SEURAT-1** public website (www.seurat-1.eu) has been an essential channel of **SEURAT-1** outreach activities. It is regularly updated with the most recent developments at the cluster level. Its main objective is to support the dissemination of information about the **SEURAT-1** Research Initiative to the large audience of stakeholders, scientists and to the general public. In addition, it also became a source of statistically important information regarding the impact of the dissemination activity.

The existing content of the website includes a general overview of the **SEURAT-1** Research Initiative, detailed information on its objectives and results, future vision and strategy, the work structure, overviews of the seven cluster projects involved and their contributions. The website has been regularly updated in line with the results of the research initiative. The 'Publications' section has been enriched with dissemination material from individual projects, such as the new **SEURAT-1** leaflet, NOTOX leaflets and film/press releases. The 'Who-is-Who' section, now a well-known depository of important information about the experts involved in **SEURAT-1**, enjoys a considerable number of visits. The 'Bibliography' currently includes a unique list of relevant articles updated on a regular basis. The website also informs visitors about forthcoming events, training activities and other important news within the cluster. In 2014 COACH will set up an online library containing all publications issued by **SEURAT-1** members. This is considered a major update to the website.

In 2013 the website also allowed COACH to evaluate the impact of the **SEURAT-1** Annual Reports. Over the course of one month, a specialised questionnaire was published on the website's homepage. The questionnaire asked visitors to respond to a few questions related to the Annual Report. Within one month, COACH gathered 60 responses out of which 19 responders belonged to industry, 17 to research, 11 to academia, three to policy makers and ten to other types of target groups. Three questions were established, with five possible responses:

- Absolutely yes
- Rather yes
- Rather not
- Absolutely not
- No opinion

A free text window was included for other recommendations.

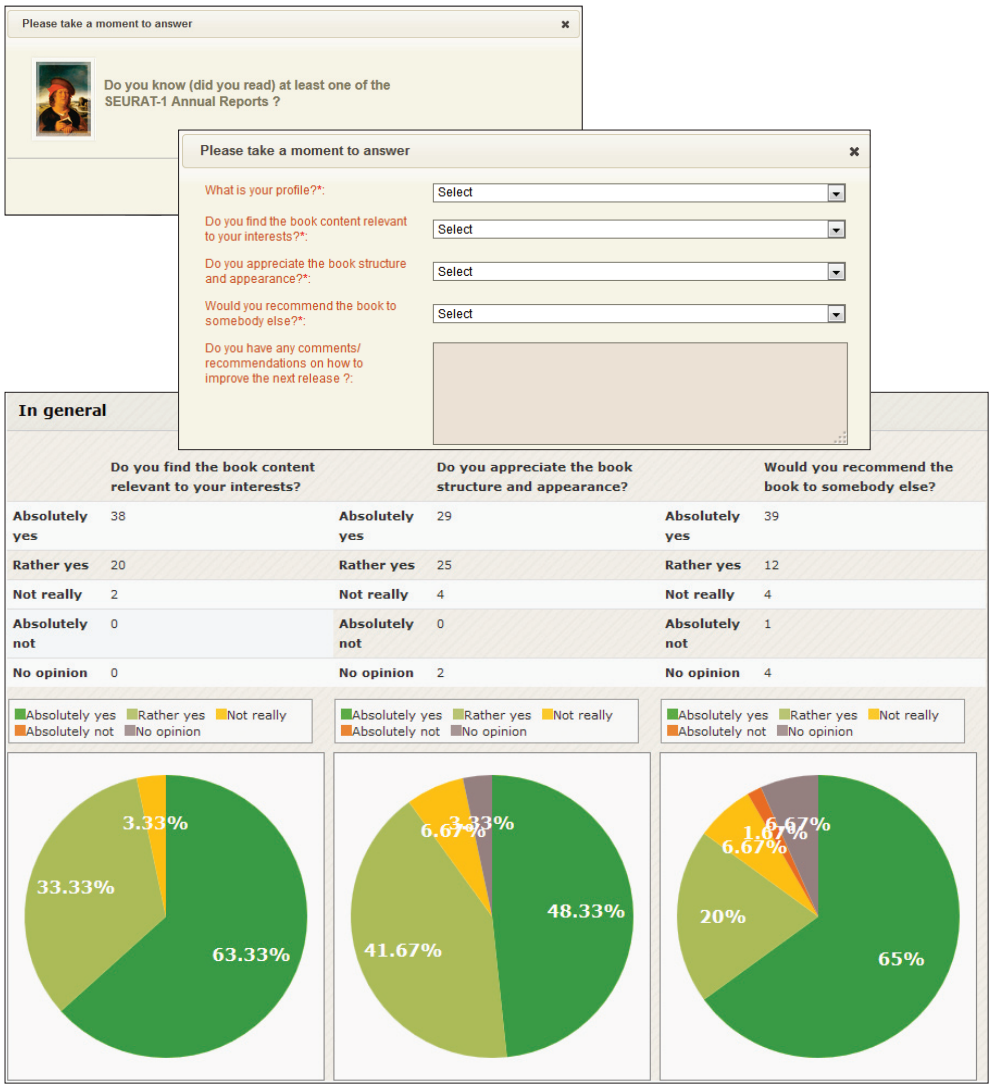
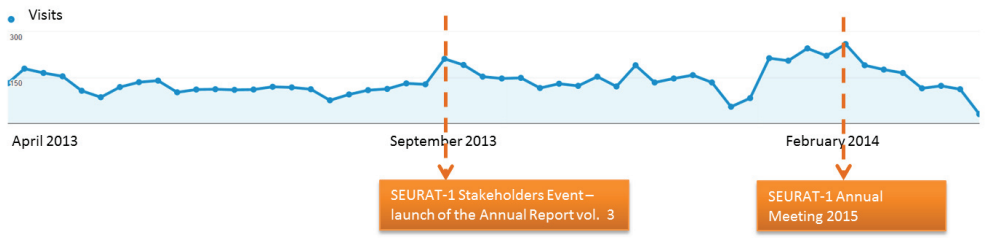


Figure 4.69 *The* ***SEURAT-1*** *Annual Report questionnaire and results.*

The detailed results are displayed in a pie chart (*Figure 4.69*) showing the distribution of profiles responding to the questionnaire. The questionnaire can hence be considered as highly relevant due to a relatively high number of responses, covering all profiles. The responses were in most cases very positive – people consider the Annual Report highly relevant to their interests; they appreciate the lay-out and they would certainly recommend it to other readers. Such results indicate that the Annual Reports represent good value for money and should continue to be used disseminating the **SEURAT-1** message on an international level, as planned at the outset of the research initiative.

As regards the numbers of visitors to the public website, the following figures (*Figure 4.70* and *Figure 4.71*) provide an overview of the visits on the website for the period from April 2013 until March 2014. The periods after the launch of the Annual Report at the Stakeholders Event and the **SEURAT-1** Annual Meeting are, as usual, the two busiest periods for the public website.



	Country / Territory	Visits	Visits
		7,215 % of Total: 100.00% (7,215)	7,215 % of Total: 100.00% (7,215)
1.	France	1,005	13.93%
2.	United Kingdom	890	12.34%
3.	Germany	869	12.04%
4.	United States	771	10.69%
5.	Italy	648	8.98%
6.	Belgium	630	8.73%
7.	Brazil	402	5.57%
8.	Japan	182	2.52%
9.	Sweden	161	2.23%
10.	Netherlands	148	2.05%

***Figure 4.70 SEURAT-1** public website statistics: timing and countries (source: Google Analytics).*

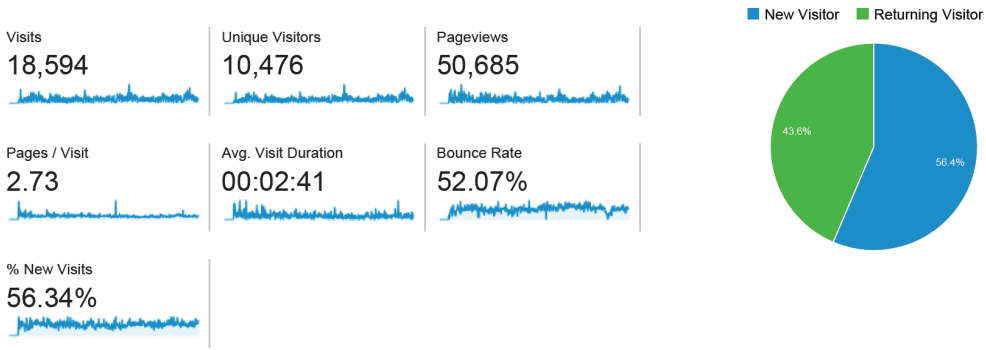


***Figure 4.71 SEURAT-1** public website statistics: total number of visits (source: Google Analytics).*

4.12.5 Other Dissemination Material

Some of the **SEURAT-1** dissemination material created in the first year has been updated and is still used, including the **SEURAT-1** leaflet, **SEURAT-1** and COACH posters and standard PowerPoint presentations. In addition to this, new publicity material was been introduced in 2012 in the form of a USB stick. This was introduced to efficiently disseminate existing **SEURAT-1** material (in particular the Annual Reports) at cluster Annual Meetings and international conferences where the shipment of printed documents would create strong logistics constraints. The introduction of the USB stick also responds to the preference of many participants not to carry too much paper back home after a meeting/conference.

Since this strategy proved to be a success, COACH decided to continue with the USB stick idea and developed a new leaflet that included the latest information on the cluster as well as the first results, with an embedded USB stick in a form of a Credit Card (*Figure 4.72*). This method of dissemination was an irrefutable success: the first release of 500 copies was exhausted within a few months. COACH is currently planning another batch of this practical material and will distribute them at upcoming events.

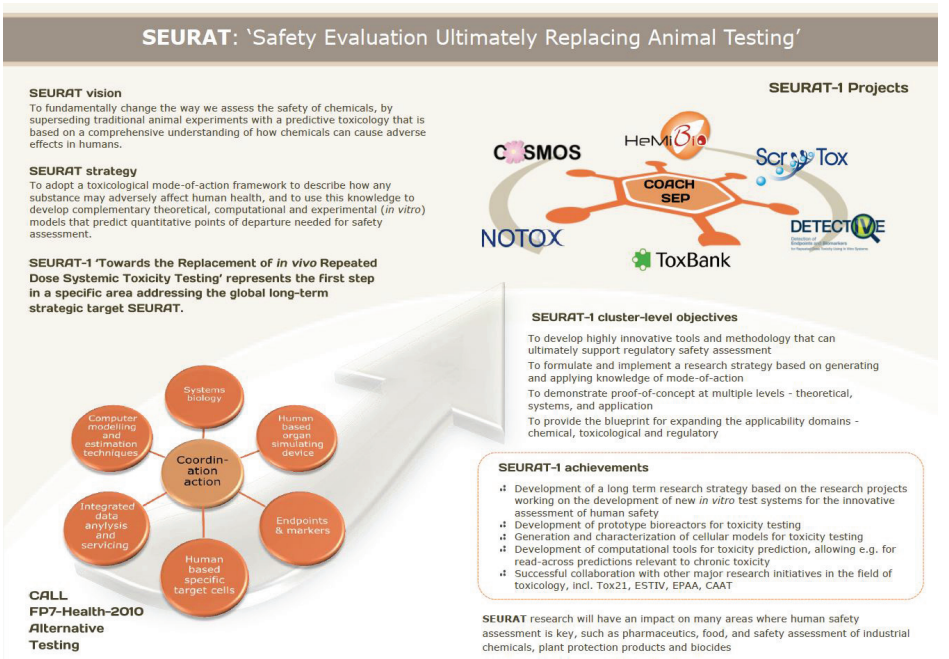
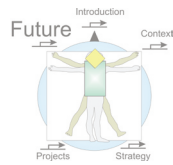


***Figure 4.72 SEURAT-1** new leaflet and embedded USB stick*



5 PREPARING FOR THE FUTURE

"We should all be concerned about the future because we will have to spend the rest of our lives there."

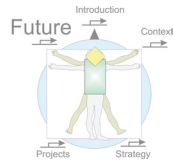
Charles F. Kettering

5.1 Introduction

Tilman Gocht, Michael Schwarz

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 needs to be addressed in the future. The efforts of setting up international collaborations culminated in a workshop held in summer 2013, which was 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. Although both initiatives have a common goal, which is the implementation of state-of-the art technologies emerging from recent scientific advances in safety assessment procedures, the approaches to doing so are fundamentally different: Tox21, not restricted to any one field within the arena of toxicology, is following a screening strategy, studying a high number of chemicals in a very diverse set of available test systems. In contrast, **SEURAT-1** focuses on repeated dose systemic toxicity and has selected a limited number of well-studied chemicals for the development of mode-of-action driven test batteries using only human cells, including reporter cell lines derived from induced pluripotent stem cells. Hence, both research programmes are highly complementary: knowledge about toxicity pathways from Tox21 inspires the construction of mode-of-action descriptions in **SEURAT-1**, and new assays developed within **SEURAT-1** may find their way into Tox21. These perspectives of liaising **SEURAT-1** with the most important US initiative in the field completes this fourth **SEURAT-1** Annual Report.



5.2 Related International Activities

Tilman Gocht, Michael Schwarz

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) or those that ended in 2013 are considered in this compilation.

5.2.1 European Activities

EU Horizon 2020: The EU Framework Programme for Research and Innovation

The European Commission's new funding scheme, Horizon 2020, combines the aspects of three separate initiatives into one single programme: It is the follow-up programme of the 7th Research Framework Programme (FP7), incorporating innovation aspects from the Competitiveness and Innovation Framework Programme (CIP) and the EU contribution to the European Institute of Innovation and Technology (EIT). In total, €80 billion in funding will be made available between 2014 and 2020.

Besides highlighting excellent science, Horizon 2020 prioritises 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 will be addressed, and the area of 'Health, demographic changes and wellbeing' was identified as one of six societal challenges on which funding will be focused (although EU support of health-related research and innovation is not limited to this particular societal challenge). 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 this will establish a new focus on innovation-related activities bridging the gap between fundamental research, and the development of new knowledge-driven products and their implementation into the market.

The work programmes for the years 2014 – 2015 were published and grouped according to the six societal challenges. The calls for proposals most relevant to the **SEURAT-1** Research Initiative are:

- PHC 33 – 2015 (draft): New approaches to improve predictive human safety testing (within the societal challenge 'Health, Demographic Change and Wellbeing');
- SFS 12 – 2014 Assessing the health risks of combined human exposure to multiple food-related toxic substances (within the societal challenge 'Sustainable Food Security');
- NMP 29 – 2014 Increasing the capacity to perform nano-safety assessment (within the societal challenge 'Nanotechnologies, Advanced Materials and Production')

International cooperation has been identified as key to success in research and innovation and, consequently, Horizon 2020 is not restricted to applicants from member states of the European Union, but instead open to participation from across the world.

More information: http://ec.europa.eu/programmes/horizon2020/

EU FP7: 7th Framework Programme of the European Union represented by the European Commission

Funding in the field of predictive toxicology within the previous European Union's funding scheme for research and innovation, FP 7, which was active until 2013, was organised within the Health Theme. Besides the SEURAT-1 Research Initiative, a number of projects are still active and are briefly described in the following:

HeCaToS *(Hepatic and Cardiac Toxicity Systems Modelling)*: HeCaToS is a collaborative large-scale integrated project funded within the European Commission's 7th Framework Programme (FP7) under the Health Theme. HeCaToS started in 2013 and will run until 2018. A total of 14 European participants from different scientific sectors (academia and industry) are working on this project. The overall goal is the development of integrative *in silico* tools for predicting human liver and heart toxicity.

The overall objective of HeCaToS is to develop an integrated framework for modelling toxic perturbations in liver and heart across multiple scales. Advances in computational chemistry and systems toxicology will be combined for this purpose and case studies based on biopsies from patients suffering from liver injuries or cardiomyopathies due to adverse drug effects will be developed. Particular attention will focus on adverse outcome pathways related to mitochondrial deregulations and immunological dysfunctions.

Scientific Coordinator: Jos Kleinjans (University of Maastricht, The Netherlands)

More information: http://www.hecatos.eu/

Envisaged cooperation: Given the focus on liver and heart toxicity and adverse outcome pathways related to specific diseases, the relevance to the **SEURAT-1** Research Initiative is obvious. A close cooperation is foreseen and the scientific coordinator of HeCaToS, Jos Kleinjans, was invited to the fourth **SEURAT-1** Annual Meeting held in February 2014, where he gave an overview presentation about the objectives of and methods used in the HeCaToS project as a starting point for identifying areas of cooperation.

ChemScreen *(Chemical Substance in vitro / in silico Screening System to Predict Human- and Ecotoxicological Effects):* ChemScreen is a collaborative project 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 (EPA) 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 in 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/

Existing collaboration: There is an overlap between the consortia of the **SEURAT-1** Research Initiative and ChemScreen (Inge Mangelsdorf, DETECTIVE/Fraunhofer Institute for Toxicology and Experimental Medicine, Germany; Michael Schwarz, COACH/University of Tuebingen, Germany). Common interests between both consortia also exist in the field of developing *in vitro* screening tools.

diXa *(Data Infrastructure for Chemical Safety):* diXa is also 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 an e-science environment) for storing multiplexed data sets, as produced by past, current and future EU research projects for 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 existing 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 between 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 offering quality assessments and newly developed tools and techniques for data management, all supported by hands-on training. Through its joint research initiative and 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/

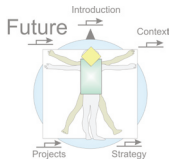
Existing collaboration: Clemens Wittwehr from the **SEURAT-1** consortium (Joint Research Centre, Ispra, Italy) is a partner in the diXa project.

AXLR8 *(Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development):* AXLR8 was 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 final report was published in December 2013 and suggested establishing a steering group as the executive body of a central coordination mechanism, which should use 'top-down' coordination for all EU research activities in the field of predictive toxicology under Horizon 2020 and beyond. Furthermore, AXLR8 has identified three pillars as structural elements for future work; each pillar contains recommendations for two objectives: pillar 1, 'AOP Discovery and Informatics' (objective 1: AOP discovery across human and environmental toxicity/ disease areas in conjunction with OECD; objective 2: EU-wide human biomonitoring and molecular epidemiology); pillar 2, 'Enabling Technologies and Infrastructures' (objective 3: *in vitro* tools for mechanistic safety assessment; objective 4: advanced computational tools for safety assessment); pillar 3, 'Implementation' (objective 5: advancement or 'orphan' *in vitro* models/strategies with regulatory applicability; objective 6: supporting regulatory application of advanced testing and assessment approaches).

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) was a member of the AXLR8 Scientific Panel. Summary reports of **SEURAT-1** activities were presented at the 2011 and 2012 AXLR8 workshops and are also part of the respective AXLR8 Annual Reports.



Predict-IV *(Profiling the toxicity of new drugs: a non animal-based approach integrating toxicodynamics and biokinetics):* Predict-IV was a collaborative large-scale integrated project funded within the European Commission's 7th Framework Programme (FP7) under the HEALTH Theme. Predict-IV started in 2008 and ended in 2013. Overall, 21 European participants from different scientific sectors (academia and industry) worked on this project. The overall goal was the development of new strategies for improved assessment of drug safety in the early stages of development and a late discovery phase.

The project was motivated by the deficit of preclinical toxicity testing approaches, which could 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 were integrated in Predict-IV to improve and optimise cell culture systems for toxicity testing. Predict-IV formed 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 were used for analysis, evaluation and integration of data from *in vitro* experiments. Additionally, Predict-IV highlighted advances in '-omics' technologies and high-content imaging and therefore increased the probability of 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 were 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 area of interest of Predict-IV that was relevant to **SEURAT-1** was the use and interpretation of '-omics' data for the identification of biomarkers for toxicity.

ESNATS *(Embryonic stem cell-based novel alternative testing strategies):* ESNATS was also funded within the European Commission's 7th Framework Programme (FP7) under the HEALTH Theme. It started in 2008 and ended in 2013. All in all, 27 European participants were involved in ESNATS. The project demonstrated 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 aimed 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.

ESNATS was divided into four key research areas, covering the following complementary scientific aspects: (i) the sub-project entitled 'Reproductive Toxicity' investigated 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' dealt with the effects of compounds on neuronal development and viability (functionality); (iii) the sub-project 'ESC-based toxicogenomics and toxicoproteomics' focused 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' concentrated 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 were also active in ESNATS (Jürgen Hescheler/University of Cologne, Germany; Jan Hengstler/IFADO, Dortmund, Germany; Vera Rogiers/Vrije Universiteit Brussel, Belgium). Mode-of-action descriptions of neurotoxicity are currently under discussion in **SEURAT-1**, and an interest of ESNAT that was relevant to **SEURAT-1** was in the fields of using '-omics' data for biomarker identification and the use of PBPK models for *in vitro* to *in vivo* extrapolation.

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 to improve the drug development process. Typical IMI consortia consist of partners from academia and industry including SMEs. IMI's research projects that are selected for funding through open calls for proposals must adhere to the four areas of the strategic research agenda: (i) predicting safety; (ii) predicting efficacy; (iii) knowledge management; and (iv) education and training. In total, three calls for proposals were launched in 2013 (9th–11th Call 2013). The most relevant projects for the **SEURAT-1** activities are briefly described as follows.

A second phase (IMI 2) is currently under discussion. If approved, it will start in 2014 and run for 10 years, with a proposed total budget of €3.45 billion. The new focus of the strategic research agenda would be 'the right prevention and treatment for the right patient at the right time' with the goal of developing next generation vaccines, medicines and treatments.

More information: http://www.imi.europa.eu/

StemBANCC *(Stem cells for biological assays of novel drugs and predictive toxicology):* StemBANCC is funded by the Innovative Medicines Initiative Joint Undertaking (IMI JU). The

project started in 2012 and will run for 5 years. In total, 35 European participants are involved in STEMBANCC with the aim of generating 1,500 high-quality human iPS cell lines from 500 people that can be used by researchers to study a range of diseases and test for drug efficacy and safety. Mainly skin and blood samples will be taken from patients with certain diseases, people who display adverse reactions to drugs, and healthy individuals. The cells will be re-programmed until they reach their pluripotent status and characterised in terms of their genetic, protein and metabolic profiles. All cell lines will also undergo a rigorous quality check. The project also investigates the use of these cell lines for toxicity testing and will generate liver, heart, neuron and kidney cells for this purpose.

A key objective of StemBANCC is to deliver a biorepositry of well-characterised human iPSCs from different disease groups. Key components in the work programme include (i) the provision of biomaterials and biodata; (ii) cellular phenotypic discovery; and (iii) assay development and validation.

Scientific Coordinator: Martin Graf (F. Hoffmann-La Roche Ltd, Basel, Switzerland)

More information: http://stembancc.org/

Envisaged collaboration: There is an obvious overlap of research interests between StemBANCC and the **SEURAT-1** project *SCR&Tox*: the common goal of both consortia is to generate well-characterised biological resources for the purpose of improved drug development (StemBANCC) and toxicity testing (both). Regarding the development of differentiation protocols, both consortia are targeting the same organs (liver, heart and the nervous system). Hence, interactions between these research groups are desirable.

MIP-DILI *(Mechanism-based integrated systems for predicting drug-induced liver injury):* Another IMI project is MIP-DILI, which started in 2012 and will run for five years. MIP-DILI brings together 26 partners from academia and industry with the aim of developing improved tools for liver toxicity testing in the early stages of the drug development process. This will require a deepened understanding of the science behind drug-induced liver injury and using that knowledge to overcome the many drawbacks of the tests currently used.

Cultures of liver cells in one-dimensional and three-dimensional configurations will be evaluated; the latter will integrate different types of liver cells to form three-dimensional units that accurately mimic human liver physiology. Natural differences between patients will be taken into account through the generation of iPS cell lines from patients who are particularly sensitive to drug-induced liver injury. The objectives of MIP-DILI are to

- Identify and validate an improved panel of *in vitro* "best practice assays" for predicting DILI in the human population;
- Explore and understand the relationship between *in vitro* assay signals and

DILI *in vivo*, in preclinical test species and in humans;

➠ Develop and validate novel systems modelling approaches that integrate multiple preclinical data types to improve prediction of DILI in humans;

➠ Enhance shared understanding of the value and limitations of new and existing approaches for DILI hazard identification and risk assessment between academia, pharmaceutical and regulatory agencies.

Project coordinator: Kevin Park, University of Liverpool, UK

More information: http://www.mip-dili.eu/

Envisaged cooperation: The importance of drug-induced liver injury within the **SEURAT-1** Research Initiative is clearly shown in the theoretical mode-of-action descriptions as part of the **SEURAT-1** proof-of-concept case studies. Liver fibrosis, cholestasis and steatosis are addressed in these case studies, and the elucidation of mechanisms as the basis for the development of toxicity testing is the focus of interest. Hence, exchanging information between the consortia would be beneficial for both and, as a starting point, Kevin Park (MIP-DILI coordinator) was invited to the **SEURAT-1** workshop 'Mechanisms underlying repeated dose systemic toxicity' held in November 2011 in Ispra.

eTOX *(Integrating bioinformatics and chemoinformatics approaches for the development of expert systems allowing the in silico prediction of toxicities):* eTOX is also funded by IMI, was started in 2010 and will run for five years. The consortium comprises 25 partners. 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 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: Francois Pognan, Novartis, Basel, Switzerland

More information: http://www.e-tox.net/

Existing 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' held in 2012. 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 eTOX could provide some support in the refinement of mode-of-action descriptions through the elucidation of additional key events. This led to a collaboration between the **SEURAT-1** project COSMOS and eTox regarding the development of computational profilers for hepatotoxicity and mining of repeated dose toxicity data.

SAFE-T *(Safer and Faster Evidence-based Translation):* Another IMI project is SAFE-T, which started in 2009 and will run for five years. Overall, 20 partner organisations are working together to improve the drug development process through the development of tools for prediction, detection and monitoring of drug-induced injuries to the kidney, liver and vascular system, using markers in patients' blood and/or urine. The ultimate goal is to identify, for each of the three organ toxicities, a set of biomarkers that are more specific, more sensitive and more predictive than those currently available, and to gain regulatory acceptance for routine use of these biomarkers in drug development.

The specific objectives are to

- Evaluate the utility of safety biomarkers for monitoring organ safety in humans;

- Develop assays and devices for clinical application of safety biomarkers;
- Gather sufficient evidence to qualify safety biomarkers in clinical drug development and in translational contexts in cooperation with the health authorities, such as the European Medicines Agency (EMA) and the US Food and Drug Administration (FDA);
- Gain evidence for how safety markers may also be used in the diagnosis of diseases and in clinical practice.

Project coordinator: Michael Merz, Novartis, Basel, Switzerland

More information: http://www.imi-safe-t.eu/

Envisaged cooperation: SAFE-T is working in a similar field to the **SEURAT-1** project DETECTIVE. Common organs in both projects are the liver and the kidney and both projects are working on biomarker identification.

Important Institutions that are active in **SEURAT-1**-related fields

NC3R^{s}: The British 'National Centre for the Replacement, Refinement and Reduction of Animals in Research' (NC3R^{s}) offers funding for feasibility studies to advance the development and application of non-animal technologies. An amount of £4 million will be made available by the UK's Technology Strategy Board (TSB), the Biotechnology and Biological Sciences Research Council (BBSRC), the Engineering and Physical Sciences Research Council (EPSRC) and the Defence Science and Technology Laboratory (DSTL). A key aim of the funding is to harness the commercial potential of technologies in this area, including biological-, tissue engineering- and imaging-related fields (e.g. cellular engineering and '-omics' technologies), manufacturing-related fields (e.g. high-throughput technologies and microfluidics) and information and communication technology-related fields (e.g. *in silico* approaches and data mining). Ultimately, the aim is to produce better tests and systems that more accurately predict efficacy, safety and environmental effects.

The competition is open to companies in the pharmaceutical, biotechnology, chemical, agrochemical, personal care and contract research industries. Several networking and partnering events to facilitate consortia-building were held until January 2014 and the deadline for applications was the end of March 2014. The applications are currently being assessed by an independent panel of experts.

More information: http://www.nc3rs.org.uk/page.asp?id=2009

Envisaged cooperation: Such feasibility studies as outlined above are highly relevant to the **SEURAT-1** Research Initiative and COACH will monitor the review process in order to contact the successful consortium to discuss options for cooperations.

CEFIC *(The European Chemical Industry Council)*: CEFIC represents 29,000 large, medium and small chemical companies in Europe and it is the forum and the voice of the chemical industry in Europe. Most importantly for **SEURAT-1** is the 'Long-range Research Initiative' (CEFIC LRI), which was established as an integral part of CEFIC's innovation strategy to improve the regulatory framework of the chemical industry in Europe. The focus is on gaps in the industry's knowledge and understanding that are critical for risk assessment. Areas were identified where scientific knowledge relevant for both the industry and the regulators should be enhanced. Funding is being made available for research in these areas through requests for proposals. The most relevant request for proposals to the **SEURAT-1** Research Initiative in 2013 was entitled 'Use of non-animal data to supplement and strengthen read-across' (code: LRI-AIMT4).

The objective of this research program is to develop non-animal approaches to supplement and strengthen read-across for mammalian toxicology end points. Integrated approaches which combine chemoinformatics (computational), *in vitro* models and/or '-omics' technologies are of particular interest. The project should be directed at complex end points of mammalian toxicity such as repeated dose toxicity, and methods for strengthening read-across arguments for these endpoints should be developed. The project should be considered as an initial proof-of-principle study for the proposed approach. The deadline for proposal submissions was 10 January 2014 and the project is expected to start in May 2014. It will run for 2-3 years.

More information: http://www.cefic-lri.org/

Envisaged collaboration: There is an obvious overlap of interest with the **SEURAT-1** proof-of-concept case studies targeting the application level in the regulatory context of safety assessment. One approach followed by the **SEURAT-1** Research Initiative is the read-across approach with exactly the same aim as outlined above. Therefore there is a strong desire to establish a collaboration with the successful applicant of the above-described project.

EURL ECVAM (*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 as well as the establishment of a specialised database service;

➠ To coordinate at the European level the independent evaluation of the relevance and reliability of non-animal 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 is intended to intensify 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.

ECHA *(European Chemicals Agency)*: ECHA is the driving force among regulatory authorities in implementing the EU's chemicals legislation for the benefit of human health and the environment as well as for innovation and competitiveness. ECHA helps companies to comply with the legislation, advances the safe use of chemicals, provides information on chemicals and addresses chemicals of concern. ECHA was founded in 2007 and is based in Helsinki, Finland. ECHA's work helps to ensure that chemicals are used safely and that the most hazardous chemicals are replaced by safer alternatives.

ECHA's most relevant field of activity for the **SEURAT-1** Research Initiative is the implementation of the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation. REACH entered into force in 2007 and was adopted not only to improve the protection of human health and the environment from the risks posed by chemicals (while enhancing the competitiveness of the EU chemicals industry), but also promotes alternative methods for the hazard assessment of substances in order to reduce the number of tests on animals.

More information: http://echa.europa.eu/

Existing collaboration: The regulatory perspective on human safety assessment of chemicals within **SEURAT-1** is ensured through the engagement of an ECHA representative in the

SEURAT-1 Scientific Expert Panel (SEP). He is actively involved in the case study planning as a co-leader of the **SEURAT-1** Safety Assessment Working Group.

OECD *(Organisation for Economic Co-Operation and Development)*: The OECD 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 use by governmental agencies 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 further testing of chemicals, based on the molecular properties that are linked to potential toxicity. High-throughput screening (HTS) using *in vitro* assays and selected chemicals are 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** feed directly into the respective current OECD activities. Furthermore, COSMOS actively contributes to the QSAR Toolbox Project through the development of approaches to group molecules for the prediction of chronic toxicity.

CAAT-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 academic sectors to address the need for human-relevant methods; (ii) make use of funds strategically 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 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 are 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

SCCS *(Scientific Committee on Consumer Safety)*: The SCCS is a part of 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/

Existing collaboration: Vera Rogiers (Vrije Universiteit Brussel, Belgium; active in the **SEURAT-1** projects *HeMiBio* and DETECTIVE) is an external expert in the SCCS working group on cosmetic ingredients.

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. EFSA plays a major role in Europe's food safety system by providing independent scientific advice and assessing all risks concerning the food chain.

More information: http://www.efsa.europa.eu/

5.2.2 International Activities

USA

Tox21: The 'Toxicology in the 21st Century' (Tox21) program is a joint initiative of the US 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), US 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 as well as 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 NCATS 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 being addressed.

Four different working groups were established within Tox21: (i) Assays/Pathways Group, which is responsible for identifying key toxicity pathways/assays, incorporating 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 patterns 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: Russel Thomas (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/Tox21/

Envisaged cooperation: A joint meeting between **SEURAT-1** and Tox21 was organised in June 2012 in Ispra, Italy (see also the workshop report in section 5.3.1). Common interests as a basis for future collaboration were discussed on this occasion and exchange activities are going to be implemented on the level of the **SEURAT-1** proof-of-concept case studies.

ToxCast™ *(Screening Chemicals to Predict Toxicity Faster and Better)*: The EPA launched ToxCast in 2007 as an important component of their 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, which is currently running, involves the profiling of approximately 800 additional chemicals, most of them with limited toxicity data as compared with phase I chemicals. Selected chemicals from a broad range of sources, including drugs, 'green' chemicals, chemicals in cosmetics and other consumer products, are being investigated in this phase.

Profiling through ToxCast means that a chemical is tested in over 800 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 the 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/

Existing cooperation: The **SEURAT-1** project COSMOS established a collaboration with ToxRefDB on mutual use of repeated dose toxicity data in the respective data bases. Further collaborations are envisaged (see entry above under Tox21).

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 communities' largest molecular toxicology reference database and informatics system. It is a current project of the 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', held in Tuebingen in 2012. 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 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 2012, the NIH issued 19 awards, 12 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.

NIH anticipates committing up to $70 million over the next five years to this initiative.

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 May 2012, ICCVAM published a five-year plan for years 2013 to 2017 with the overall aim to better align ICCVAM and NICEATM with the vision laid out by the National Academy of Sciences in the 2007 NRC Report *Toxicity Testing in the 21st Century: A Vision and A Strategy*, while simultaneously fulfilling the mission of ICCVAM to implement the 3Rs of toxicity testing (i.e., replace, reduce, and refine) in accordance with the ICCVAM Authorization Act of 2000. The initial steps towards this new strategic direction are to: (i) set priorities and identify areas for scientific focus for immediate resource investment (i.e. investment into projects where there is a high likelihood of success within a reasonable timeframe of 1-5 years for implementation into regulatory use, such as acute oral and dermal toxicity testing or skin sensitization); (ii) to develop plans to improve communications between stakeholders and the public (e.g. through focused workshops); and (iii) to explore new paradigms for the validation and utilisation of alternative toxicological methods.

More information: http://iccvam.niehs.nih.gov/

PSTC *(Predictive Safety Testing Consortium)*: 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 FDA in 2005. The PSTC provides a platform for pharmaceutical companies to share and validate each other's safety testing methods with consultation from the FDA, its European counterpart, the European Medicines Agency (EMA), and the Japanese Pharmaceutical and Medical Devices Agency (PMDA). Since 2013, PSTC collaborates with the IMI project SAFE-T (see above) on the development of important new drug safety tests.

The mission of PSTC is to identify new and improved safety testing methods and submit them for formal regulatory qualification by the FDA, EMA and PMDA. Currently, the PSTC has 19 corporate members with the same goal: to find improved safety testing methods. The members share their internally developed methods and test these methods developed by one another across the consortium. Ten EMA and twenty-eight FDA scientists serve as advisors along with more than 250 participating scientists. C-Path leads the collaborative process and collects and summarises the data.

Executive Director: John-Michael Sauer (Critical Path Institute, Tucson, USA)

More information: http://c-path.org/programs/pstc/

HESI *(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 develops scientific programmes through committees that organise, support and execute projects, including collaborative laboratory studies, development and analysis of databases as well as workshops and conferences. The goal is always to address and reach consensus on scientific questions that have the potential to be resolved through creative application of intellectual and financial resources.

Executive Director: Syril Pettit (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 3R's 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.

The Doerenkamp-Zbinden Foundation (DZF) and CAAT are collaborating to establish the Transatlantic Think Tank for Toxicology (t^4). t^4 prepares and/or commissions high-quality analyses of toxicological problems and orchestrate workshops, reports, and review papers designed to bring to fruition the innovative approaches outlined in the report of the National Academy of Science (*Toxicity Testing and Assessment in the 21st Century*).

More information: http://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 Work 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 Work 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.

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).

The original agreement was expanded in March 2011 to include the South Korea in the ICATM.

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

EPAA *(European Partnership for Alternative Approaches to Animal Testing)*: The EPAA is a collaboration between the European Commission, European trade associations and companies from several industrial sectors. The vision of EPAA is the replacement, reduction and refinement (3Rs) of animal use for meeting regulatory requirements through better and more predictive science. Consequently, EPAA is active in research as well as in regulation. In the field of regulation the goal of EPAA is to improve the implementation of 3Rs in European regulatory testing and decision-making. In the field of research, EPAA is exploring opportunities to prioritise, promote and implement future research in the field of the 3Rs.

Furthermore, the EPAA organises an annual conference and workshops supporting the development of alternative approaches to animal testing. The 2013 annual conference was entitled 'More predictive safety science for a more competitive Europe' and was held in Brussels on 13 November 2013. Most important for the **SEURAT-1** Research Initiative was the organisation of the first **SEURAT-1** stakeholder event in September 2013, which presented latest success stories in non-animal methods for human safety assessment of chemicals and was used to launch the third **SEURAT-1** Annual Report.

More information: http://ec.europa.eu/enterprise/epaa/

ecopa *(european concsensus-platform for alternatives)*: Similarly, 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/

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* (ESTIV) is the 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 European countries. ESTIV2014 was 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 was 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 conference was held in conjunction with the second **SEURAT-1** Summer School.

The ESTIV2014 congress had an attractive scientific programme that focused on the motto 'Making sense of *in vitro* methods'. Emphasis was specifically on how new technologies can strengthen the interpretation and application of *in vitro* methods in toxicological research and risk assessment. Session themes were:

- 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.

More information: http://www.estiv2014.org/

Mondial Research Group meeting on Reduced Animal Testing

Date: 24–25 July 2014

Location: Zurich, Switzerland

Although most 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/

9th World Congress on Alternatives and Animal Use in the 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 was 'Human Science in the 21st Century'. During the past two decades, responsible reduction in the number of animals used for scientific purposes and development and improvement of 3R-relevant research methods clearly demonstrate the commitment of the scientific community to provide novel science of higher quality. Advanced experimental study designs involve completely new concepts based on pathways in cells and tissues of human origin to satisfy the highest scientific criteria in humans, the species of interest.

Main themes of the congress for discussion were:

- New Technologies (including virtual tissue models, high throughput screening models, novel 3D models, bioreactors, high-content imaging, and others);
- Predictive Toxicology – updates, computational approaches, risk assessment and advances in specific assessments (including repeated dose toxicity, pathway approaches in toxicology, systems biology, computational modelling and chemoinformatics, risk assessments, and others)
- 3Rs in Academia and Education;
- Communication, dissemination and data sharing;
- Efficacy and safety testing of drugs and biologicals;
- Human relevance;
- Ethics;
- Refinement and welfare;
- Global cooperation, regulatory acceptance and standardisation;

More information: http://www.wc9prague.org/

OTHERS IN THE FIELD

41st Annual Meeting of the Japanese Society of Toxicology (JSOT)

Date: 2–4 July 2014

Location: Kobe, Japan

The continual integration of new knowledge and technology within the field of toxicology, has created a growing need for greater scientific precision during investigations that could contribute to the protection of people from various chemical hazards. JSOT annual meetings have been a forum for meeting participants to convey their latest scientific achievements in the field of toxicology. Continuing along this line, the main theme for the 41st Annual Meeting was the 'Translation from Basic to Applied Research'.

Main themes for discussion included:

- Safety evaluation of vaccines;
- Toxicomics;
- Progress of microRNA studies in toxicology research;
- Laboratory animal welfare and animal models: International issues, trends and challenges;
- Application of toxicogenomics and future perspectives;
- JSOT collaborating symposium with JSIT: New development of innovative immunotoxicological investigations opening up for the next generation of scientists.

More information: http://jsot2014.jp/index_en.html

Workshop: Genetic Toxicology at the Crossroads: From Qualitative Hazard Evaluation to Quantitative Risk Assessment

Date: 10–11 July 2014

Location: Lancaster, UK

This satellite workshop was organised in the context of the European Environmental Mutagen Society conference (EEMS), held at the same venue from 6–10 July 2014. There is increasing awareness within the genetic toxicology community that qualitative hazard-based approaches should move towards quantitative risk-based methodologies to facilitate data interpretation in the context of informing human risk. Given that genetic toxicologists employ a number of different *in vitro* as well as *in vivo* test systems, it is imperative that approaches for comparing

the dose-metrics across the test systems be standardised so that a point of departure (POD) or no-observed-genotoxic-effect-level (NOGEL) derived in one test systems can be extrapolated or compared to another and eventually progress from experimental models to humans.

This workshop brought together experts in the fields of genetic and general toxicology, risk assessment, and computational biology, representing industry, academia, and government to address and make recommendations on a path forward on this topic, including the identification of any key data gaps in our knowledge that require further research. While the focus of this workshop was on genetic toxicology studies, the key outcomes of this workshop will have a much broader impact across various toxicology disciplines.

Main themes for discussion included:

- Comparing PoD metrics across test systems and endpoints: tools and case studies;
- *In vitro* to *in vivo* extrapolation: tools and approaches for the evaluation and extrapolation of exposure across test systems;
- Recommendations and current initiatives for the use of dose response data for risk assessment: different approaches.

More information: http://www.hesiglobal.org/i4a/pages/index.cfm?pageID=3647

50th Congress of the European Societies of Toxicology

Date: 7–10 September 2014

Location: Edinburgh, Scotland, UK

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). The 2014 conference aim is to 'Advance Science for Human and Environmental Health'.

Main themes for discussion include:

- Regulatory toxicology biomarkers;
- Human and environmental risk assessment;
- Emerging *in vitro* models;
- Computational toxicology;
- '-Omics' technologies;

- Mechanisms of toxicity;
- Organ toxicities.

More information: http://www.eurotox2014.com/

OpenTox Euro 2014

Date: 22–24 September 2014

Location: Athens, Greece

The OpenTox Euro series of meetings emerged as a continuation of the EU-funded Health-FP7 project 'OpenTox' which was completed successfully in August 2011. The ambitious plan is to further develop OpenTox as an infrastructure and community with annual events held in Europe and the USA in the area of predictive toxicology and related fields.

The OpenTox Euro 2014 meeting will place particular emphasis on industrial applications and challenges of predictive toxicology as well as on risk assessment and regulatory acceptance of *in silico* approaches, including read-across and weight-of-evidence. Special sessions are planned on metabolism and systems biology and on predicting the toxicological effects of engineered nanomaterials.

More information: http://www.douglasconnect.com/event/opentox-euro-2014

OpenTox USA 2014

Date: 18–19 November 2014

Location: Baltimore, USA

See short description above (OpenTox Euro 2014).

More information: http://www.douglasconnect.com/event/opentox-usa-2014

54th Annual Meeting of the Society of Toxicology (SOT)

Date: 22–26 March 2015

Location: San Diego, 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.

More information: http://www.toxicology.org/AI/MEET/AM2015

51st Congress of the European Societies of Toxicology

Date: 13–16 September 2015

Location: Porto, Portugal

The motto of the EUROTOX 2015 is 'Bridging Sciences for Safety'. Conference topics include:

- Toxicology biomarkers;
- Alternative methods in toxicology;
- Predictive toxicology;
- Safety assessment of mixtures;
- Regulatory toxicology.

More information: http://www.eurotox2015.com/

5.3 SEURAT-1 Meets Tox21

Elisabet Berggren

5.3.1 Workshop Report

For the first time, principal scientists from the two largest research initiatives in the USA (Tox21, see section 5.2.2) and the EU (**SEURAT-1**) in the field of animal-free safety assessment were brought together for a three-day workshop (25-27 June 2013) to discuss opportunities for cooperation. A limited number of participants from each project were invited to assist during the workshop. The event in Ispra (Italy) was hosted by the European Commission's Joint Research Centre and facilitated a detailed exchange of ideas and results covering topics of mutual interest such as human-relevant *in vitro* methods, ultra-high throughput screening, bioreactors for tissue based testing, chemo-informatics, biophysical modelling for toxicity prediction, and reference chemical selection for system development and validation. Time was also dedicated to discussing efforts underway in developing decision-making frameworks for chemical safety assessment using novel data sources that address different regulatory needs.

The format of the workshop comprised five different sessions, as described below. The key outcome of the information exchange and discussion was a comprehensive list of cooperation topics at both the technical level, including the sharing of research materials, such as data,

cells, assays, computational models, and at the application level, by teaming up on predictive toxicity and safety assessment proof-of-concept case studies.

Details of the workshop's sessions were:

- An introduction to ToxCast, Tox21 and **SEURAT-1** research programmes in terms of their strategic aims, structure, research focus and selected highlights. Opportunities for cooperation between the USA and EU in the area of health related research in the framework of Horizon 2020 were also explored;
- Description of the chemical inventories assembled byToxCast, Tox21 and **SEURAT-1** and the different approaches employed for chemical selection;
- 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 upon. Attention was given to the rationale used to select the assays and the mechanistic information derived from them;
- Review of 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;
- Best exploitation of the output and efforts for the purposes of chemical safety assessment, exploring how data from 'non-standard' (non-animal) methods can be used in different decision-making contexts and frameworks.

SEURAT-1 partners showed a large interest in the ToxCast data (Phase I and II) that is planned to become publicly available during the latter part of 2013 via the EPA-NCCT website, including both *in vitro* HTS data and *in vivo* animal data (ToxRefDB). Colleagues from the US EPA volunteered to assist **SEURAT-1** partners in searching for data. Since the database became available several of the **SEURAT-1** case studies used ToxCast data when identifying additional chemicals to test. ToxCast/ToxRefDB data was also used to complement the COSMOS database. In relation to data issues, it was agreed that ToxBank, DETECTIVE, NOTOX and the **SEURAT-1** Mode-of-Action Working Group should establish collaboration with Tox21 colleagues on meta-analysis and mining of '-omics' data. Such a working group was also established after the meeting.

In terms of chemicals selection it was suggested that chemicals screened within ToxCast or Tox21 could be followed up in secondary 'orthogonal' testing within **SEURAT-1**, for example for mitochondrial toxicants. *HeMiBio* volunteered to further test a smaller set of chemicals in their bioreactor systems (liver). It was also said that chemicals in the ToxCast or Tox21 10k libraries could be made available for testing in **SEURAT-1** systems, if the requested chemical

set is sufficiently large (>100) and the data will be shared with the Tox21 consortium and eventually put in the public domain. In addition, the **SEURAT-1** gold compounds so far not included in the Tox21 library should be included.

Tox21 representatives expressed an interest in identifying candidate assays being developed within **SEURAT-1** compatible with ultra-HTS (i.e. 1536 well-plate format) that could be considered for inclusion within the Tox21 screening programme.

The **SEURAT-1** working groups assisted in setting up collaborations in many aspects: Firstly, all partners wanted to identify AOP's of common interest. The **SEURAT-1** Mode-of-Action Working Group volunteered to assist in looking for overlaps. Tox21 colleagues active in AOP development should join this working group. Linked to the AOP issues was also the idea to exchange knowledge and experience on systems (e.g. templates, vocabulary) for describing *in vitro* methods/assays (meta-data) that would be necessary for building up prediction systems. Furthermore, it was agreed to establish information exchanges on PBBK modelling and biokinetics. Tox21 colleagues should join the **SEURAT-1** Biokinetics Working Group. Also information exchange on biophysical toxicodynamic modelling was encouraged. Finally, relevant Tox21 colleagues should join the **SEURAT-1** Safety Assessment Working Group to get involved in safety assessment discussions, such as the design of Integrated Assessment Frameworks that exploit non-standard (non-animal) data, and the two proposed case studies underpinning the safety assessment proof-of-concept within **SEURAT-1**.

On a more general basis it was encouraged that all partners should consider scientific staff visits and short stays at partner facilities. It was agreed that this would be a very efficient means for mutual learning and for establishing good working relationships.

Follow-up initiatives to **SEURAT-1** in the field of safety assessment that may be supported under Horizon 2020 were agreed to include a strong international component, which would be beneficial both for a faster and more efficient development of chemical assessment based on alternative methods as well as facilitate future collaboration in between **SEURAT-1** and Tox21 partners.

5.3.2 Transatlantic Cooperation to *Advance in vitro* Methods in Safety Science using High Throughput Screening Technology

On Monday 14 January 2014, representatives from EURL ECVAM and the US NCATS at the NIH met at the NCATS's site in Bethesda (MD, USA) to review progress and plan future engagement in relation to their formal Collaboration Agreement (in place since July 2012).

The collaboration was motivated by a common goal of exploiting (ultra)-high-throughput and high content screening (HTS/HCS) platforms based on advanced robotics and imaging technologies to advance the development, validation and utilisation of *in vitro* cell-based

methods for the safety assessment of chemicals used in a variety of sectors. In particular, the collaboration is aimed in part to support the Tox21 initiative, and the **SEURAT-1** Research Initiative.

At the NCATS' NIH Chemical Genomics Center (NCGC), scientists are using the centre's high-throughput screening robotic system to test Tox21's unique library of 10,000 compounds (composed of environmental chemicals and approved drugs) in a comprehensive set of *in vitro* assays. Measuring the activity of a compound across a range of HTS/HCS cellular and biochemical assays can help determine its potential to disrupt biological pathways that may result in toxicity and adverse health effects in certain conditions. EURL ECVAM has been operating its own HTS/HSC facility for several years, albeit on a smaller scale. This facility generates toxicity data on carefully selected sets of reference chemicals to expedite the development and validation of cell-based *in vitro* methods that demonstrate potential for application in regulatory safety assessment.

The joint work plan foresees cooperation on using *in vitro* HTS/HCS methods to explore how chemicals can affect the mitochondria (the energy production components of cells) and how this may lead to adverse effects in humans, for example in the liver, heart or brain. Another area of cooperation will be in the performance assessment of HTS/HCS assays designed to indicate a chemical's potential to interact with the hormone system. Comparing results obtained from assays using different cell types and read-outs but which address similar biological effects will help identify the strengths and limitations of each method and thus facilitate its optimum use in HTS/HCS screening batteries. EURL ECVAM will also act as a European focal point to identify and evaluate novel assays being developed within EU research consortia that could be tailored for implementation on the NCATS HTS/HCS platforms to generate data on the 10k Tox21 library.

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.

ALF Acute liver failure

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.

CET Cryo-electron tomography

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

DNEL Derived no effect level

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

EM Electron microscopy

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

GO Gene Ontology

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

HPC Hepatic progenitor cells

HSC Hepatic stellate cells

HSEC Hepatic sinusoidal endothelial cells

hSKP human skin-derived precursors

HTS High-Throughput-Screening

IATA Integrated Assessment and Testing Approaches. Combination of approaches in a weight of evidence (see WoE) as a rational integration of tests data and predictions coming from various data domains (e.g., *in silico* models, computational chemistry, high content and high throughput bioassays, genomics, human exposure, pharmacokinetics, etc.) in order to better understand the likely biological targets of chemicals.

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.

KEGG Kyoto Encyclopedia of Genes and Genomes or KEGG is a collection of online databases dealing with genomes and enzymatic pathways. The database was created to improve understanding of functions and utilities of the biological systems, such as the cell, the organism and the ecosystem, from molecular-level information, especially large-scale molecular datasets generated by genome sequencing and other high-throughput experimental technologies. Further information and access to the databse: http://www.genome.jp/kegg/.

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.

LOAEL Lowest Observed Adverse Effect Level

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

OED Oral Equivalent Dose. The dose which results in *in vivo* concentrations corresponding to the *in vitro* effective concentration of interest.

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.

PoT Pathway of Toxicity. See 'Toxicity Pathway'.

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.

TOR Threshold of Regulation. A concept adopted by the US Food and Drug Administration (FDA) to exempt from the requirement of a food additive listing regulation any substance used in food-contact substances (e.g., food-packaging or food-processing equipment) that migrates, or that may be expected to migrate, into food, if it becomes a component of food only at levels that are below the threshold of regulation. Specifically, an identified migrant of known chemical structure can be exempted if the incremental dietary concentration is below 0.5 μg/kg of diet and the substance has not been shown to be a carcinogen in humans or animals. If the FDA is satisfied that the conditions for exemption are met, the chemical does not ordinarily have to undergo toxicological testing, nor the formal pre-market safety evaluation by the agency.

Toxicity Pathway According to the Report from the US National Research Council (NRC) 'Toxicity Testing in the 21st Century: A Vision and a Strategy' 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.

WoE Weight of evidence. A quantitative method for combining evidence in support of a hypothesis.

ZFN-HR Zinc finger nuclease homologous recombination

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)

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- 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 fourth 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.