EUROPEAN COMMISSION
Research & Innovation

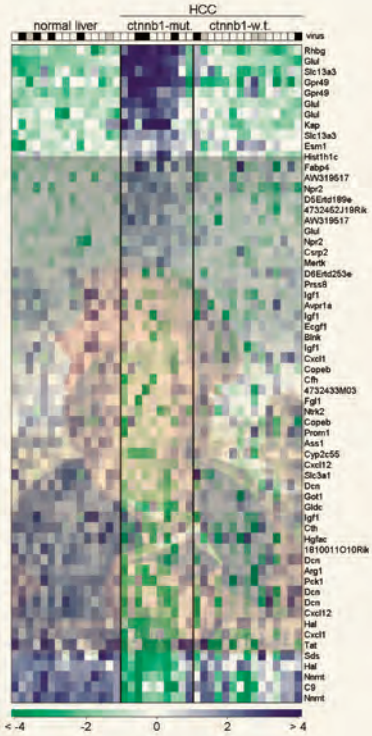
Cosmetics Europe
the personal care association

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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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 fifth 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"

Mechanism-Based Methods for Improved Toxicity Testing

Edited by:
Tilman Gocht, Michael Schwarz

volume 5

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

For **SEURAT-1**, 2015 might be the most interesting and challenging year as projects are coming to an end and sponsors and stakeholders alike will be keen to learn about tangible results as indicators for 'return on investment'. The **SEURAT-1** books are an excellent tool to take stock but the final outcomes will also need to be in the peer reviewed publications to form the basis for follow-up on promising methodologies to further build the toolbox on animal free testing. It is also expected that **SEURAT-1** will be providing sufficient ground for setting the strategy of next research programmes on alternatives for systemic toxicity.

The **SEURAT-1** symposium on December 4th 2015 will be a showcase to learn more about the extensive research efforts during the last 5 years and how these can be translated into solutions for safety assessment ultimately replacing animal testing. Another important objective of the symposium is to put the **SEURAT-1** outcomes into context of other related on-going and future initiatives from the EU and the US as co-ordination and sharing of research will also contribute to making progress in the field.

For any successful economy, science, research and innovation is core. For Cosmetics Europe they are essential and key drivers for maintaining the industry's competitive position and securing its license to operate in a global market. The industry also needs to be able to build upon solid risk-based regulation making best use of the current scientific knowledge. Ultimately science will be the only and ultimate solution to overcome the need for animal testing for proving safety of product ingredients and chemicals.

Marketing safe products is the highest priority of the industry. However, current safety assessment and regulatory practices are still largely depending on animal testing which in time is hindering innovation in the industry. Given the ban no new animal testing will be forthcoming. On the positive side there is the wish and the scientific capabilities to improve safety assessment approaches based on alternatives. It is our belief that in time research on alternatives can provide us with better safety assessment tools in terms of predictability (ie. more relevant for assessing human risk) at a faster pace and at lower costs. This will help better, faster and more competitive innovation.

SEURAT has and still will make in our view a substantial contribution on the journey away from traditional animal testing and towards a mechanistic understanding of key toxicological events based on (human) cell responses, a comprehensive understanding of bioavailability and systems biology. This already has helped to improve Read Across and TTC approaches and to pave the way for *ab initio* approaches.

Another learning from **SEURAT-1** is that case studies will help establish, validate and implement the test tool box and we recommend that programmes following **SEURAT-1** will build on this experience.

The industry is committed to continue the journey and is optimistic that the availability of new technologies will help to better understand the complex chains linking early molecular events at cellular level with organ and systemic effects. The ability to link multiple data streams and to translate non-animal (*in vitro* or *in silico*) findings in quantitative way to *in vivo* is essential to drive a paradigm shift in chemical safety testing to meet regulatory requirements.

In our view **SEURAT-1** and following projects will only be fully successful if the scientific efforts are accompanied by continuous exchange with stakeholders worldwide, including the regulatory community, validating agencies, interested public and academia as well as partners for commercial exploitation (small and medium sized enterprises and large industries) to maximise its impact.

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Executive Summary

The **SEURAT-1** Research Initiative invites stakeholders from industry, policy, science and regulatory authorities to attend the final **SEURAT-1** Symposium on 4 December 2015 at the Square Meeting Centre in Brussels, Belgium. The **SEURAT-1** achievements and their impact in the field of human safety assessment will be highlighted at this symposium, demonstrating how the extensive research efforts of **SEURAT-1** during the last 5 years can be translated into solutions for safety assessment ultimately replacing animal testing (see Text Box 1). A research strategy was formulated as a backbone for the development of innovative *in vitro* and *in silico* tools that can be combined to form integrated approaches to testing and assessment, addressing the needs of regulatory authorities for acceptance of animal-free toxicity testing methods.

Major Achievements of the SEURAT-1 Research Initiative

Development of a research strategy based on generating and applying knowledge of mode-of-action;

Development of highly innovative tools and methodology that can ultimately support regulatory safety assessment, compiled in a Tools & Methods Catalogue;

Sustainable collection of data and standard operation procedures in a Data Warehouse;

Development of a conceptual framework to combine evidence derived from predictive tools to support a safety assessment decision in a biologically-rational manner;

Application of the developed tools and concepts in case studies addressing three scenarios:

- The read-across case study demonstrating immediate use of *in vitro* and *in silico* tools in regulatory toxicology;
- The *ab initio* case study, illustrating proof-of-concept of how a risk assessment for a cosmetic ingredient might be done without animal testing;
- The Threshold of Toxicological Concern (TCC) case study, expanding the applicability domain of the established TTC approach towards cosmetic ingredients applied dermally.

The history of the **SEURAT-1** Research Initiative is strongly influenced by the full ban on animal testing for cosmetic products within the European Union, which finally entered into force on 11 March 2013. 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 fifth 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 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.

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. 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.

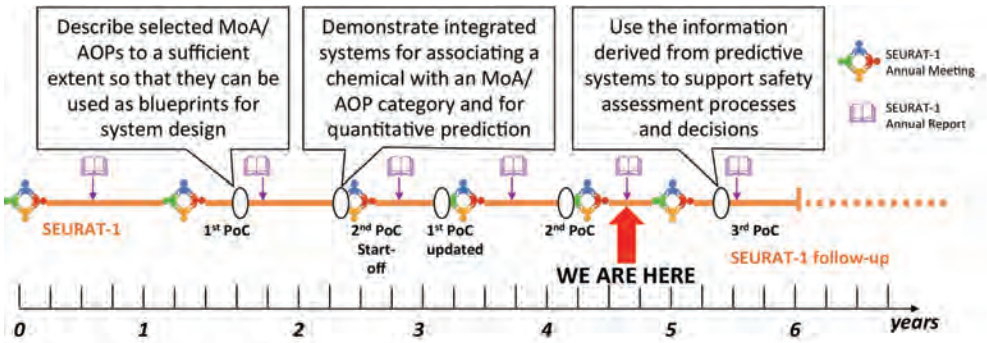

Figure 1 *Roadmap illustrating the timing of the proof-of-concept (PoC) at three conceptual levels as the backbone for interactions between the* ***SEURAT-1*** *projects.*

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 fifth 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) progress reports from the proof-of-concept case studies at the three above-mentioned levels. As shown in *Figure 1*, the focus has moved from the level of test system development based on already existing mode-of-action descriptions towards the application level. Three different scenarios were developed on the application level, to which methods developed within **SEURAT-1** will contribute (see Text Box 2). It is expected that the case studies will also provide guidance towards further development of mechanism-based integrated toxicity testing strategies and modern safety assessment approaches beyond **SEURAT-1**.

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 volume.

(ii) Regulation: 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 and this is the clear focus of this Annual Report. The most advanced field regarding implementation of non-animal approaches into safety assessment is skin sensitization and lessons learned from this toxicological endpoint are summarised in this volume. The situation of assessing skin sensitization by using non-animal test methods is: (i) the full AOP is known; (ii) *in silico*, *in chemico* and *in vitro* methods are available for different events of the AOP; (iii) the individual test methods have been combined into different testing strategies and the prediction models of such testing strategies are being evaluated. All of these issues together result in the opportunity to assess skin sensitization with a testing strategy combining different methods in an integrated approach to testing and assessment (IATA), which is also the focus of **SEURAT-1** in the field of repeated dose systemic toxicity. However, in the absence of accepted methods for many endpoints there is an increasing demand for the development of frameworks that incorporate existing knowledge and complement it with new data, preferably based on non-animal methods. **SEURAT-1** developed such a conceptual framework and other initiatives are also active in this field. In a separate section we present a brief overview about the framework developed by the Health and Environmental Sciences Institute (HESI) called RISK21 roadmap, which has a clear focus on problem formulation as a starting point and suggests a tiered approach for both exposure and hazard assessment.

(iii) Science: This year's Annual Report provides an overview of the emerging discipline called Green Toxicology. The basic idea of this concept is to move the safety assessment schemes to the beginning of the production cycle of a chemical or a product, i.e. to the molecular design. Initially developed for chemistry ('Green Chemistry'), principles of toxicology can be implemented into the enterprise of designing safer chemicals. With this perspective, the unique emphasis of the Green Toxicology approach is in using twenty-first century toxicology tools as a preventative strategy to 'design out' undesirable human health and environmental effects, thereby increasing the likelihood of launching a successful, sustainable product. Finally, we present reflections on public–private partnerships as a funding scheme for research in the field of alternatives to animal testing. This model was applied to **SEURAT-1** and lessons learned from an industrial perspective focusing on project management issues are given in a separate section.

In the first three volumes of this Annual Report, 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. The fourth volume of the Annual Report marked a transition to the concrete demonstration of how these mode-of-action descriptions trigger the development of tools for toxicity testing, through the formulation of case studies on the methodological level. This fifth Annual Report summarises initial results from the case studies operating on this methodological level and presents the consolidated plans to use them in the context of safety assessment, considering the three different application scenarios outlined in Text Box 2. Altogether, the intention is to demonstrate 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.

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 Adverse Outcome Pathway 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 molecular modelling approaches to predict potential binding to nuclear receptors involved in the development of liver steatosis Development of biomarker based on a read-across use case on valproic acid analogues 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 Threshold of Toxicological Concern (TTC)

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 were 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 are being 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. 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 three typical safety assessment scenarios; (i) the objective of the first case study is to use the **SEURAT-1** methods in the context of ‘read-across’, that is, to demonstrate that, using **SEURAT-1** methods, information 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; (ii) the objective of the second case study is to arrive at a point of departure for a particular chemical, that can be used as a basis for safety decisions by conducting an *ab initio* assessment using the new methods developed within the **SEURAT-1** Research Initiative. Piperonyl butoxide was chosen as the test compound with a hypothetical use scenario defined for a skin cream and a shampoo. The liver is the target organ for piperonyl butoxide, even though the mechanism of action is unknown. Furthermore, the two **SEURAT-1** standard reference compounds methotrexate and valproic acid (VPA) were selected as positive controls for fibrosis and steatosis; (iii) the objective of the third case study is to extend the concept of the Threshold of Toxicological Concern (TTC) to dermal exposure. Initially developed for oral exposure, methods were developed within the **SEURAT-1** Research Initiative to allow the application of the TTC concept in the context of dermal exposure and the goal of this case study is to confirm that the approach is practical and yet scientifically sound enough to be useful for both regulatory bodies and industries.

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 fourth 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 generation of pluripotent stem cell-derived hepatocytes for toxicity testing. A differentiation protocol was developed and the stem cell-derived hepatocytes were thoroughly

characterised and compared with benchmarking hepatocyte cell models. 3D spheroids were prepared and used for repeated dose toxicity studies.

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

HeMiBio reports on progress in the integration of sensors into the *HeMiBio* bioreactor for the non-invasive monitoring of the metabolic status of the *in vitro* liver tissue model. Furthermore, real-time high-sensitivity measurements of oxygen uptake were used for toxicity testing and uncovered a novel mechanism of acetaminophen toxicity. In addition, a master cell line was created, allowing the generation of newly targeted pluripotent stem cell lines equipped with toxicology readout cassettes within two weeks, without the need for single cell isolation. Finally, the results from the genome-wide profiling of promoter DNA methylation in primary, non-cultured liver cell types (hepatic stellate cells, hepatocytes and sinusoidal endothelial cells) suggests a differential epigenetic programming of these cell types in the liver.

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

The DETECTIVE report focuses on progress in three different fields: (i) genetically modified proximal tubule cells were generated and now represent an almost unlimited cell source for high-throughput kidney toxicity screening; (ii) further characterisation of the *in vitro* liver model developed in the DETECTIVE project revealed proper bile canalicular architecture in the hepatocyte cell systems, which could represent novel biomarkers predicting cholestasis in *in vitro* compound screening; (iii) the generation of a BAC-GFP reporter platform of toxicity pathways based on BAC engineering of the human hepatoma HepG2 cells has been extended, including reporters for stress pathways that are closely related to the manifestation of the steatosis AOP.

Delivery of an integrated suite of computational tools to predict the effects of long-term exposure to chemicals in humans and support safety assessment.

COSMOS reports on the implementation of the models into transparent and adaptable KNIME workflows to make the models developed in the COSMOS project publicly and freely available. They include biokinetics models, models for absorption, structural alerts and models for toxicity, as well as tools for chemical space analysis. The additional WebPortal

versions are easily executable through a web browser even for a non-proficient user of the software and are linked to documentation and user guidance. Together with the COSMOS Database, the COSMOS KNIME models constitute a true legacy of COSMOS, which will be usable beyond the end of the project and provide tools to support chemical safety and risk assessment.

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.

The focus of the NOTOX report is on a valproic acid case study. Multi-omics data of a joint experiment applying valproic acid (VPA) on 2D HepaRG cultures were generated and clear trends were observed in viability, transcriptomic, proteomic, metabolomic and epigenetic analyses. An initial physiologically based pharmacokinetic (PBPK) and cellular mechanism of action coupled model was generated for valproic acid. Parallel experiments using 3D spheroids of HepaRG cells to study effects of VPA over a period of two weeks were conducted and it was found that cell types remain constant irrespective of drug application but EC_{50} decreased with time, confirming earlier experiments.

ToxBank

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

ToxBank has further developed tools and support mechanisms/processes for the population of its data warehouse. The report highlights options for integrated data analyses combining **SEURAT-1** data with those from other research programmes such as the US ToxCast initiative. Most ToxBank data warehouse support operations, including uploading of protocols and other documents as well as searching and downloading records, have been implemented and self-help material is available on the ToxBank website. The ToxBank consortium agreed to the sustainable maintenance of access to all ToxBank reference data generated on the **SEURAT-1** programme as an OpenTox resource until at least 2020.

COACH

Cluster-level coordination and support action.

The COACH report provides information about the cluster-level coordination activities (i.e. the third strategic review consisting of a SWOT analysis and an update of the **SEURAT-1** roadmap), the facilitation of exchanges between the projects, and dissemination of research activities at the cluster level. The main COACH activities in 2014 were (i) the organisation of a planning group, which published recommendations for

follow-up research initiatives in *Archives of Toxicology* (see below); (ii) the further development of the dissemination strategy with a focus on reaching out to the public through reports in the mass media.

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. An overview of the **SEURAT-1** roadmap, highlighting the contributions of the individual projects to the achievement of cluster-level objectives, is presented together with an outline of the **SEURAT-1** Tools & Methods Catalogue 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 were 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 co-operate with the various complementary international research programmes on the way 'towards the replacement of *in vivo* repeated dose systemic toxicity testing' and various collaborations have been set up since the start of the **SEURAT-1** Research Initiative. As this research programme is now nearing its completion, planning a possible follow-up initiative becomes an important task. A planning group consisting of members from the **SEURAT-1** Scientific Expert Panel was established for this purpose and published recommendations for future research in the field of predictive toxicology. These recommendations, focusing on the translation and implementation of available *in vitro* and *in silico* tools into regulation as well as on the development of a new, mechanism-based toxicological hazard assessment framework, finalises this fifth **SEURAT-1** Annual Report.

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1 INTRODUCTION

Tilman Gocht, Michael Schwarz

'The larger the island of knowledge, the longer the shoreline of wonder.'

Ralph W. Sockman

Background

On 11 March 2013 the full ban on animal testing for cosmetic products came into force. From this date, animal testing for marketing of new cosmetic products in the European Union was 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. This was due to unresolved questions related to the complex cellular mechanisms involved (*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 as systemic toxicity testing is 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 that 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 modes-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 by 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 T.D. 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 administration for the **SEURAT-1** Research Initiative and to the SEP. Support to the cluster is provided either directly through COACH or through the SEP.

An example of the scientific management and coordination is the development of a roadmap for the cluster as a whole (*Figure 1.1*). 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 published in the third volume of this book series. Relevant case studies addressing these three proof-of-concept levels were formulated subsequently and reported in the fourth volume. Updates and results from these case studies will be presented in this fifth volume. The overall approach for the implementation of this roadmap was developed by COACH in close cooperation with the project coordinators, and was subsequently endorsed by the SEP.

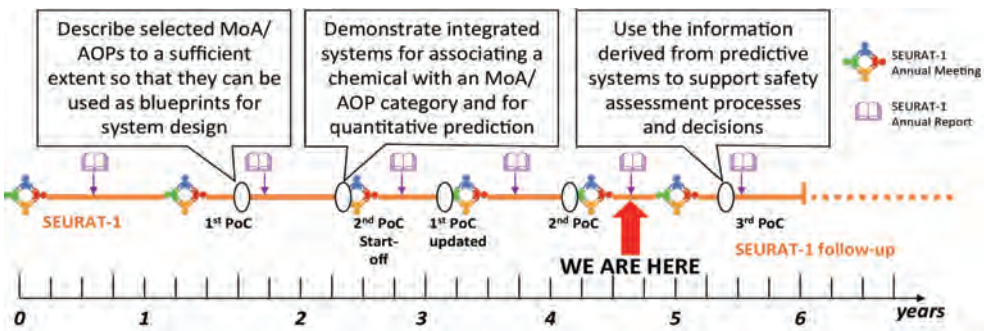

Figure 1.1 SEURAT-1 *roadmap illustrating the timing of the proof-of-concept (PoC) at three conceptual levels as the backbone for interactions between the* ***SEURAT-1*** *projects.*

The Annual Report: Something about 'Pathways'

This is the fifth 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 focused 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 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 and 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: '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 fifth volume we further elaborate on the cluster-level case studies that 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. In addition, we specify in further case studies how the data and information derived from the tools and methods developed within **SEURAT-1** can be used in safety assessment frameworks and scenarios, addressing *ab initio* predictions, read-across scenarios and predictions using a Threshold of Toxicological Concern approach. 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 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.

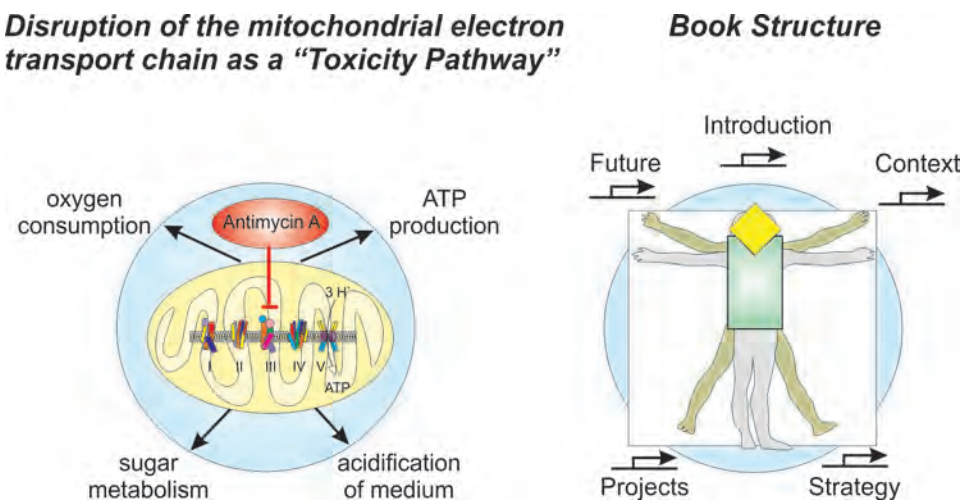

Figure 1.2 *The concept of 'Toxicity Pathways' (left panel) is mirrored by the book structure (right panel). Here we show cellular consequences of poisoning the mitochondrial electron transport chain by the model compound Antimycin A.*

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 proportion of SMEs participating in **SEURAT-1** is high, at more than 30%.

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

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

Participant	Institution / Country	Project
Project Coordinators		
Marc Peschanski	INSERM/UEVE 861, I-STEM/AFM, Evry / France	*SCR&Tox*
Mark T.D. 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	**Swedish Toxicology Sciences Research Center (Swetox), 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	U.S. Environmental Protection Agency, National Center for Computational Toxicology, 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, 2014*). The most relevant call for **SEURAT-1** activities, entitled 'New approaches to improve predictive human safety testing' (call identifier PHC-33-2015), was published on 23 July 2014 in the area 'Improving health information, data exploitation and providing an evidence base for health policies and regulation' (*European Commission, 2014).* The overall aim is to improve the efficiency of predictive toxicological testing to address key areas of concern for human health and to meet regulatory requirements (e.g. EU legislations on REACH, cosmetics, biocides). The objectives are 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 call highlights the importance of international collaboration with similar initiatives in the USA and elsewhere. The deadline for proposals was 24 February 2015 and the successful consortium will very likely start respective activities from the beginning of 2016. The relationship of this new project to the scope of the **SEURAT1** Research Initiative is obvious and underlines the importance of continuing joint efforts to accelerate the paradigm shift in toxicity testing from empirical *in vivo* studies to mechanism-based approaches that combine *in vitro* tests with *in silico* methods.

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2 CONTEXT

Science is wonderfully equipped to answer the question 'How?' but it gets terribly confused when you ask the question 'Why?'

Erwin Chargaff

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 European Union (EU). In 2009 an EU testing ban for cosmetic ingredients came into force with an extension of three specific areas: repeated dose toxicity (includes skin sensitisation, carcinogenicity and sub-acute/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 the marketing of new cosmetic products in the EU was prohibited from this date.

This chapter outlines the recent developments in the legal, regulatory and scientific context of the **SEURAT-1** Research Initiative. As the legal framework is clearly defined since March 2013 the focus of this chapter is now on the implementation into regulations of innovative approaches using non-animal data into regulation. Under this perspective, public-private partnerships may play an important role and the first section of this chapter summarises some principles to consider and lessons learned from **SEURAT-1** that may be helpful when applying this funding scheme into the forefront in future research programmes that address the development of alternatives to animal testing.

The second contribution discusses the most advanced field regarding the implementation of non-animal approaches into safety assessment, namely skin sensitization. The situation of assessing skin sensitization by using non-animal test methods is: (i) The full Adverse Outcome Pathway (AOP) is known; (ii) *in silico*, *in chemico* and *in vitro* methods are available for the different events of the AOP; (iii) the individual test methods have been combined into different testing strategies and the prediction models of such testing strategies are being evaluated. All of these issues together result in the option to assess skin sensitization with a testing strategy combining different methods in an integrated approach to testing and assessment (IATA). Currently the availability of non-animal test methods to analyse different AOP key events of the skin sensitization process is not the limiting factor. Rather, clarification is needed on which data are essential for hazard assessments (particularly for regulatory purposes) and further also for risk assessments. Lessons learned from these efforts in the field of skin sensitization may be transferable (at least in parts) to other endpoints such as repeated dose systemic toxicity.

However, in the absence of accepted methods for many endpoints there is an increasing demand for the development of frameworks that incorporate existing knowledge and complement it with new data, preferably based on non-animal methods, for the purpose of a full risk assessment. The **SEURAT-1** Research Initiative generated an approach (summarised

in section 4.10.9), but at the same time such frameworks are also being developed within other projects. This is in the focus of the third contribution, which outlines the basic considerations and principles of a framework developed by the Health and Environmental Sciences Institute (HESI), called the RISK21 roadmap. This framework has a clear focus on problem formulations as a starting point and suggests a tiered approach for both exposure and hazard assessment. Depending on the available data, the risk assessor can either make a decision regarding the safe use of a chemical, or identify data gaps that can guide the design of further experiments. As other frameworks, the RISK21 roadmap has a high degree of flexibility that allows the incorporation of new methods such as *in vitro* test systems or *in silico* calculations.

In a broader context, safety assessment schemes could also be moved to the beginning of the production cycle of a chemical or a product, i.e. to the molecular design, and this is at the heart of the emerging discipline Green Toxicology. We finalise the chapter regarding the context of the **SEURAT-1** Research Initiative this year with an overview of a framework for integrating the principles of toxicology into the enterprise of designing safer chemicals. The unique emphasis of the Green Toxicology approach is in using 21st century toxicology tools as a preventative strategy to 'design out' undesired human health and environmental effects, thereby increasing the likelihood of launching a successful, sustainable product.

2.2 The Rationale for Public-Private-Partnerships in the Context of EU Supported Research through 'Framework' Programmes - An Industry Perspective

Rob Taalman

2.2.1 Introduction

A compelling argument offered for establishing public private partnerships (PPPs) is to create a collaborative environment to maximise use of collective knowledge and take advantage of cross-disciplinary expertise among government, academic, and industry researchers – an enhancement that should, in principle, help to solve complex research problems such as systemic toxicity alternatives.

Possible benefits include maximising resources and sharing risks (primary financial but also

sharing the burden of possible failure) across partner organisations. Clearly, however, the European Commission as the organising body is challenged to address a number of issues when merging public and private interests in a common partnership. PPPs are positioned at the interface of the different roles which public and industrial research play in society. Academic research is mainly tasked to lay the foundation for society as a whole to thrive; it thus focuses on fundamental research. Complementarily, industrial research strives for transferring the findings of basic research into commercially viable fields. This is why these two kinds of research traditionally employ distinctively different principles. While academic research needs to follow a topic to its very fundaments and explore, with a great degree of curiosity, totally new grounds, industrial research must exert more discipline towards an agreed upon objective and thus manages programmes with strict milestones and goals. Uniting both research philosophies under one project roof is therefore an ambitious undertaking with promising prospects if inherent risks are mitigated. These include: definition of tangible overall goals and milestones, success monitoring, scientific metrics, partner roles/accountability and work assignments, membership equality, power balance, conflicts of interest and research objectivity, partner compatibility, partner sharing and commitment, academic/civil society inclusion, flexibility and transparency, and consideration of a special coordinated action effort to enable communication across projects, disciplines and partners.

The **SEURAT-1** Research Initiative (2010-2015), jointly supported by the EC and the Cosmetics Industry is, as the single largest PPP in the field of alternatives to animal testing, a good example of a PPP with from the onset the ambition to take advantage of all the benefits of a PPP (as listed above). How successful it has been in achieving that is addressed in some detail in the following sections.

2.2.2 Background: The Need for a Joint Research Programme

The EU had adopted the Cosmetics Directive (*Anonymous, 1976*), which provides the regulatory framework for the phasing out of animal testing for cosmetics purposes. The same provisions are contained in the Cosmetics Regulation (*Anonymous, 2009*), which replaced the Cosmetics Directive as of 11 July 2013.

Specifically, it establishes:

- Animal testing ban – prohibition to test finished cosmetic products and cosmetic ingredients on animals;
- Marketing ban – prohibition to market finished cosmetic products and ingredients in the EU which were tested on animals.

These measures were taken in full acknowledgement that a sufficient complement of non-animal alternative methods to animal testing methods/approaches for safety assessment

purposes would not be available at the time of implementation and that considerable effort was and is required to develop such methods/approaches and be accepted at the regulatory level.

Still, research and development (R&D) into alternatives can only make further progress if it can rely on multidisciplinary cooperation to solve complex scientific problems that are characteristic of this field. Failure to act is neither acceptable for European consumers (free choice and innovative products) nor for European competitiveness (jobs and growth) nor for the European Commission and the European Cosmetics industry (ensuring the safety of consumers), but developing alternatives for safety assessment is costly, involving many steps and responsible bodies until regulatory acceptance is achieved.

This is why the European Cosmetic Industry with its proven long-term track record of being able to develop non-animal alternatives to regulatory acceptance and the European Commission with its long-term ability to initiate and overlook large research programmes teamed up to conceive and run **SEURAT-1** as the largest-ever initiative in the field.

It was also known that **SEURAT-1** could only be a starting point 'Towards the replacement of *in vivo* Repeated Dose Systemic Toxicity Testing' and therefore would not provide the ultimate answer.

2.2.3 Considerations for Public Private Partnerships in the Field of Alternatives to Animal Testing

Key Drivers

In many countries there is a wish to reduce the reliance on experimental animals for testing the safety of (consumer) products. In some regions like in the EU this has resulted in regulatory requirements (e.g. the Cosmetics Regulation banning animal testing for Cosmetics product/ingredients) and the animal welfare directive (aspiration to reduce animal use throughout following the 3R principles). Also there is an expectation by the public to take measures to reduce the use of experimental animals, the confidence in science to come up with animal free solutions for safety testing and in addition a professional drive by risk assessors for continuously improving safety assessment approaches (reducing uncertainty, increasing predictability, faster and less expensive).

At the same time, there is an apparent reluctance to change from the traditional animal based safety assessment to alternative approaches – arguments are: why change an approach that has served the 'regulatory and safety assessors community' well and which is solidly anchored in various national and international regulatory schemes?

While many alternative methods (for a limited number of toxicological end points) are available and are to an extent accepted, replacing animals for assessment of systemic toxicity is a big

challenge and one that requires a change in mind-set in toxicology. Also, the traditional one to one replacement concept does not apply to systemic toxicity. Rather a set of various different alternative test methods combined with complementary risk assessment approaches (such as read across or thresholds of toxicological concern, etc.) are necessary to address complex safety questions involving several body organs and tissues. Knowledge of underlying modes-of-action, systems biology and computational methods needs to be integrated into research programmes as well as the ability to extrapolate from effect level concentrations in the *in vitro* systems to equivalent *in vivo* exposures at the appropriate level (systemic circulation, tissue). With this need of paradigm shift, traditional 'validation' schemes need to be revisited and the goal of regulatory acceptance needs to be an integral part of designing and executing research from the onset.

Another key need is the sharing of data and the accessibility of databases among various stakeholders. The ability to integrate multiple data streams is of critical importance for the systemic toxicity challenge and without a framework enabling this to happen in a controlled environment, cooperation will be hampered.

The Initiative for a PPP Lies with the Public Sector

Partnerships on research in the area of safety assessment methodologies and basic understanding of underlying processes (cellular, organ and organism level) will not necessarily evolve in a commercial environment, nor can it be achieved by the public sector alone. It can only come about through public cooperation, where the various players (academia, industry, scientists, safety assessors and regulators) share resources, data and expertise, while ensuring that the fruits of their collaboration are shared, risks and costs reduced and productivity increased. The creation of such a risk-benefit-sharing environment will reduce the failure rate and those carrying out the research will have a greater incentive to achieve their goals as the relevance to society is more obvious and the impact therefore wide (beyond their own interest group).

The right of the EU to act in this field is provided under Article 187 of the Treaty on the functioning of the EU, authorising the setting up of 'joint undertakings or any other structure necessary for the efficient execution of the European Union research, technological development and demonstration programmes'.

Measures at EU level to support trans-national and cross-sector cooperation between firms on strategic research agendas will help to establish 'critical mass', in particular through joint agenda setting, mobilisation of additional funding and increased leverage of industrial R&D investment.

The achievements of the **SEURAT-1** joint undertaking has produced a number of important results:

➠ First methods for a tool box addressing systemic toxicity (see section 4.10.2);

- Toxicity database for cosmetic ingredients TTC

- 2D/3D liver model; knowledge on AOP (liver – fibrosis; steatosis; cholestasis); tests and toxicity markers (liver, kidney, CNS, heart);

➠ Framework for safety assessment and proof-of-concept case studies for a mechanism-based safety assessment approach (see section 4.10.9);

➠ External collaboration with other large scale, key initiatives such as ToxCast (see sections 5.2.1 and 5.2.2);

➠ Considerable leverage effect on industrial R&D investment by virtue of a € 25 million contribution from the European Commission and a contribution in kind of € 25 million from the European Cosmetics industry (Cosmetics Europe);

➠ Enhanced cooperation – the **SEURAT-1** joint programme brought together large-scale industry, small and medium sized enterprises and research organisations from across the European Union; joint production of comprehensive strategic research agendas and coordination of actions with regulatory bodies and other stakeholders;

➠ Enhanced visibility and ownership of non-animal alternatives research – the **SEURAT-1** joint programme has contributed to broadening the ownership base for research on alternative methods for safety assessment.

2.2.4 The Lessons Learnt from SEURAT- 1

Despite these achievements, evaluation of the implementation of SEURAT revealed a number of improvement points:

➠ a review process of such a 5-6 year research programme both in terms of content and execution needs to be introduced (min. bi-annually) with possibilities to refine, adjust or even redirect (e.g. where there is unintended duplication or where certain approaches seem to be futile);

➠ monitoring and evaluating achievements of the various work packages and milestones needs to be improved - a process to check alignment with the overall strategic research objectives needs to be included;

➠ horizontal coordination needs to be strengthened (e.g. possible synergies between work packages should be identified and fully exploited);

- internal and external communication needs to be strengthened (in particular to policy makers and the public – laymen type of communication needs more attention).

The latter is of particular importance in the context of the aforementioned PPP as an animal testing ban being imposed despite lack of full non-animal alternative falsely implies that no animal experimentation is necessary anymore to establish the safety of ingredients or chemicals. Hence an open clear and easy to understand communication from policy makers and industry to the public is necessary to illustrate the huge challenges ahead and the research effort required to close the scientific gap towards the ultimate goal: overcoming the need for animal testing for safety evaluation of chemicals and products.

For industry to be interested in future alternatives to animal testing research PPP's, a PPP should be:

- Providing structures to facilitate partnerships along the entire research cycle, such as from early method development up to regulatory acceptance of the alternative approaches in an effective innovation-driven collaborative setting focusing on optimising alternatives research to ideally support evidence-based regulation of product and ingredient safety;
- Reducing the fragmentation of research and innovation and increase the leverage of private-sector spending in Europe;
- Developing and implementing a strategic research agenda and setting clear objectives in a pan-European structure and beyond with the necessary critical mass and budget, thereby increasing the probability of success;
- Managing research project proactively through regular review and where necessary redirect in a way that adds value for all partners but still aligned with the overall strategic objectives;
- Allowing expansion of work packages via additional funding and/or through adding partners with specific skills and expertise (late joiners); possibility to decrease programme parts that turn out not delivering as planned;
- Reducing administrative burden for applicants and participants, and funded entities such as academia and small and medium sized enterprises that would benefit from administrative simplification;
- Establishing mechanisms for cross-project co-ordination to identify synergies and taking maximum benefit for the effective execution of the strategic agenda;
- Monitoring and evaluating the overall PPP programme and individual PPP projects via a set of common key performance indicators (for both public- and

private-sector research goals), to enable the assessment of whether the PPP is achieving its objectives. This should be the responsibility of the Governing Body (e.g. steering committee) and Advisory Body (e.g. science board) as both will be overseeing the work of the 'programme management office';

➠ Arranging of a 'coordinated action' from the onset serving as a third-party convener (independent broker) to organise and facilitate PPP meetings to clarify rules, establish operational guidelines, and specify funding arrangements etc. and to ensure transparent internal (between the PPP partners and project leaders) and external (with stakeholders including the public) communications.

2.2.5 Conclusion

SEURAT-1 has been the single largest PPP in the field of alternatives to animal testing. It has paved the way towards future alternative safety assessment approaches. As expected from such pioneer work, the involved parties learn on the way. The proposed principles offered above are in no strict chronological order and are listed not to critique the execution of the SEURAT PPP but have to be seen as a pragmatic wish list to help increase the effectiveness of future PPP's.

It is clear that the efforts to create a wide-ranging collaborative effort in the form of a PPP with the aim to have a significant positive impact on health safety research (in particular on alternatives to animal testing) is not a one-size-fits-all proposition and requires a comprehensive commitment from cradle to grave (fundamental research up to regulatory acceptance of non-animal safety assessment approaches). However, given the broad agreement (PPP) cited here, it also seems clear that the effort to create general principles is likely to be helpful. The realities confronting science and public health make this imperative.

The idea is that, at a minimum, there will be further critical engagement on the presently proposed principles among key public and private stakeholders going forward.

References

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2.3 Regulatory Use of Non-Animal Test Methods in Chemical Industry: The Example of Skin Sensitization

Daniel Urbisch, Naveed Honarvar, Annette Mehling, Robert Landsiedel

2.3.1 Regulatory Requirements

In the regulatory context, the skin sensitization potential of a substance has been evaluated in the past decades using animal tests (explained in more detail below in section 2.3.3) either according to OECD test guideline (TG) 406 (i.e. the guinea pig maximisation test (GPMT) & Buehler test) or in OECD TG 429 (i.e. the murine local nymph node assay, LLNA). More recently, non-radioactive variants of the LLNA were adopted as OECD TGs 442A and 442B (also compare *Basketter et al, 2012; Kolle et al., 2012*).

Under REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals; *Anonymous, 2006*), all chemicals have to be evaluated for skin sensitization unless they are handled under strictly controlled conditions. For the REACH phases 1 and 2 (phase-in substances with an annual production volume of ≥1000 or ≥100 t/a, respectively), the skin sensitizing properties of approximately 3700 and 3000 chemicals, respectively, were assessed (*Angers-Lostau, 2011; ECHA, 2014*). It is estimated that several thousand chemicals more will need to be evaluated during phase 3 (phase-in substances with an annual production volume of ≥1 t/a) with the deadline for registration submissions being in 2018. In Annex XI of the REACH legislation (*Anonymous, 2006*) it is specified that prior to initiating animal testing, the available human, animal and alternative data should be assessed and if this data is not conclusive, in general the LLNA should be performed. The LLNA is currently the 'first-choice' method with respect to the regulation of chemicals (*Anonymous, 2006*) and agrochemicals (*Anonymous, 2014*).

Annex XI of the REACH regulation specifies how non-animal data can be used, e.g. in a weight of evidence approach, to allow an evaluation of the toxicological properties of the substance (*Anonymous, 2006*). In this context, a weight of evidence approach can be defined as the process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning a property of the substance (*ECHA, 2010*). REACH Annex XI also provides information about replacing animal testing, if validated and adequately documented *in vitro* methods are available.

In contrast to the REACH Regulation, which foresees animal testing (as a last resort), the European Union imposed an animal testing ban on both cosmetic products and their ingredients with the 7th amendment to the Cosmetics Directive (now the Cosmetics Regulation;

Anonymous, 2009). This animal testing ban was accompanied by a concurrent marketing ban, if animal tests were conducted after this date for the purpose of this regulation. This regulation therefore led to a complete phasing out of animal testing in the cosmetics sector in Europe and the development and use of non-animal test methods for new cosmetic substances is now essential.

2.3.2 New Approaches Based on the Adverse Outcome Pathway (AOP) for Skin Sensitization

With respect to the 3Rs (i.e. Replacement, Reduction and Refinement; *Russel & Burch, 1959*) numerous animal and non-animal test methods such as the LLNA, *in silico*, *in chemico* and *in vitro* test methods were developed in the past decades and some were formally validated. Most of the current test guidelines for non-animal test methods directly address a given toxicological endpoint (i.e. the adverse outcome) and not the underlying mechanisms[1] (i.e. key events of an AOP) leading to the adverse outcome itself. In general, an AOP is the sequence of causally linked events that begin with the molecular initiating event (MIE; possibly being affected by the physico-chemical properties of a substance) and lead to an adverse organism response (*Ankley et al., 2010*; *OECD, 2015*).

Skin sensitization is one of the first adverse effects which is currently being evaluated for regulatory purposes using different non-animal test methods along the AOP. To enable testing for regulatory purposes, three questions have to be answered: (i) How may the AOP for skin sensitization be described? (ii) Which non-animal test methods may adequately address the key events of the AOP? (iii) How may the results of the individual test results be used for hazard assessment?

2.3.3 The AOP for Skin Sensitization

Defining the AOP for Skin Sensitization

Skin sensitization is the first adverse outcome for which the OECD has fully described an AOP (*OECD 2012a; b*) and in which four key events were identified. The MIE in the AOP for skin sensitization is the formation of an immunogenic hapten-protein complex and thus described by protein/peptide reactivity of a substance - key event 1. This event is followed by the two cellular events ‘keratinocyte activation’ and ‘dendritic cell activation’, representing key event 2 and 3, respectively. The first three key events may subsequently lead to an organ response (i.e. T-cell proliferation in the lymph node - key event 4) und eventually to an adverse outcome affecting the organism (i.e. allergic contact dermatitis upon repeated contact to an allergen).

1 i.e. the OECD TGs 437 and 439 for eye and skin irritation testing or assays addressing mutagenic or genotoxic effects (such as OECD TGs 471, 473 and 476)

Methods Addressing Key Events of the AOP for Skin Sensitization

A number of promising non-animal test methods covering AOP key events for skin sensitization have been developed (e.g. reviewed in *Goebel et al., 2012; Mehling et al., 2012*). The direct peptide reactivity assay (DPRA; *Gerberick et al., 2004*) is an *in chemico* method which serves to evaluate peptide reactivity (AOP key event 1). The KeratinoSens™ (*Natsch & Emter, 2008*) and LuSens (*Ramirez et al., 2014*) assays are surrogates for keratinocyte activation (key event 2), whereas the human cell-line activation test (h-CLAT; *Ashikaga et al., 2006*) and U937-based tests (*Ade et al., 2006; Bauch et al., 2011; Piroird et al., 2015*) are surrogates for dendritic cell activation (key event 3). A number of non-animal test methods are well advanced and have undergone (or are undergoing) the formal validation need for regulatory acceptance. Since February 2015, OECD TGs are available for the DPRA (OECD TG 442C; key event 1) and ARE-Nrf2 luciferase test methods (e.g. KeratinoSens™; OECD TG 442D; key event 2). For the h-CLAT, an OECD draft TG has been prepared, which is expected to be adopted by the end of 2015. In addition, several further *in vitro* test methods using reconstructed tissue models or *in vitro* test methods with gene expression read-outs have been developed in the past decade (*Mehling et al., 2012*). Furthermore, *in silico* tools based on current mechanistic knowledge on peptide reactivity such as electrophilicity (e.g., DEREK, OECD QSAR Toolbox, TIMES-SS), or statistical evaluations of structural fragments determined from the model training set (e.g. Case Ultra, Vega, TOPKAT; *Teubner et al., 2013*) may be used for assessing skin sensitization.

Feasibility of the Use of Non-Animal Test to Identify Skin Sensitization Hazard Potential for Regulatory Purposes

One single non-animal test method is probably not sufficient as a 'stand-alone' method to cover the endpoint skin sensitization. Therefore, the use of a testing strategy including a combination of non-animal tests methods which ideally cover different AOP key events will be necessary (*Basketter et al., 2013*). Results from non-animal test methods may be used in a weight of evidence approach as defined in section 2.3.1. Nevertheless, to date, there is no consensus on the amount of data necessary to fulfil the information requirements enabling a reliable hazard assessment: Is assessment of the MIE alone sufficient, or should further key events be evaluated? Does dermal penetration and biotransformation – both events are not defined to be key – also have to be tested as part of a testing strategy?

Regulatory accepted animal testing methods directly address either the sensitization phase (LLNA; OECD TG 429 and its modified versions OECD TGs 442A and 442B) or the adverse outcome itself (guinea-pig based tests; OECD TG 406) and provide assessment rules or prediction models to translate their results into hazard assessments in terms of decision-

making. To date, the extent of testing needed and the overall assessment of the results obtained from non-animal approaches can only be decided on a case-by-case basis taking into account the respective weight of evidence; a pre-defined and regulatory accepted strategy on how to link the different test results for a hazard assessment is yet not available. In other words: Even if all individual non-animal test methods that have gained regulatory acceptance (see previous section) are performed, a hazard assessment based on these methods may not be sufficient for regulatory purposes (whereas an animal test such as the LLNA (see section 2.3.1) will suffice). Hence, hazard assessments based upon the results obtained from non-animal test methods addressing key events of the AOP for skin sensitization – instead of the adverse outcome itself – remain a challenge.

An Example of a Testing Strategy - '2 out of 3' Weight of Evidence (WoE) Integrated Testing Approach

The '2 out of 3' weight of evidence integrated testing approach is probably one of the most straightforward testing strategies for skin sensitization hazard identification with data available for over 200 substances. It represents a deterministic and comprehensible prediction model combining results from three non-animal test methods which can be used to assess skin sensitization potential based on a weight of evidence pertaining to three different AOP key events including the MIE. The combination of test methods used (i.e. 1. DPRA; 2. LuSens or KeratinoSens™; 3: h-CLAT or (m)MUSST) covers the first three of the four AOP key events. The decision 'positive' or 'negative' for each considered non-animal test method is based on the decision criteria developed and used for the respective test method. Any two congruent results of the three non-animal test methods considered for this prediction model rule the overall assessment: If at least two of the three non-animal test methods are positive, the substance is considered a skin sensitizer. Conversely, a substance having at least two out of three negative results is considered a non-sensitizer (also compare *Figure 2.1*). This approach considers all key events of the AOP essential for the progression towards the adverse outcome, but the assays addressing the key effects overlap some key events (e.g. cysteine reactivity in DPRA and ARE-Nrf2 assays). To facilitate acceptance of the '2 out of 3' prediction model by both the regulatory and scientific communities, *in chemico* and *in vitro* results for 213 substances have been qualitatively compared to both, animal and human data, where available. The '2 out of 3' prediction model achieved accuracies of 90% (n = 101) or 79% (n = 180) when compared to human or LLNA data, respectively. These results indicate that the '2 out of 3' prediction model predicts human data more accurately than LLNA data. In addition, the LLNA itself achieved an accuracy of only 82%, when compared to the human data of this set of substances. In conclusion, the '2 out of 3' prediction model exceeds the predictivity of the LLNA (*Urbisch et al., 2015*).

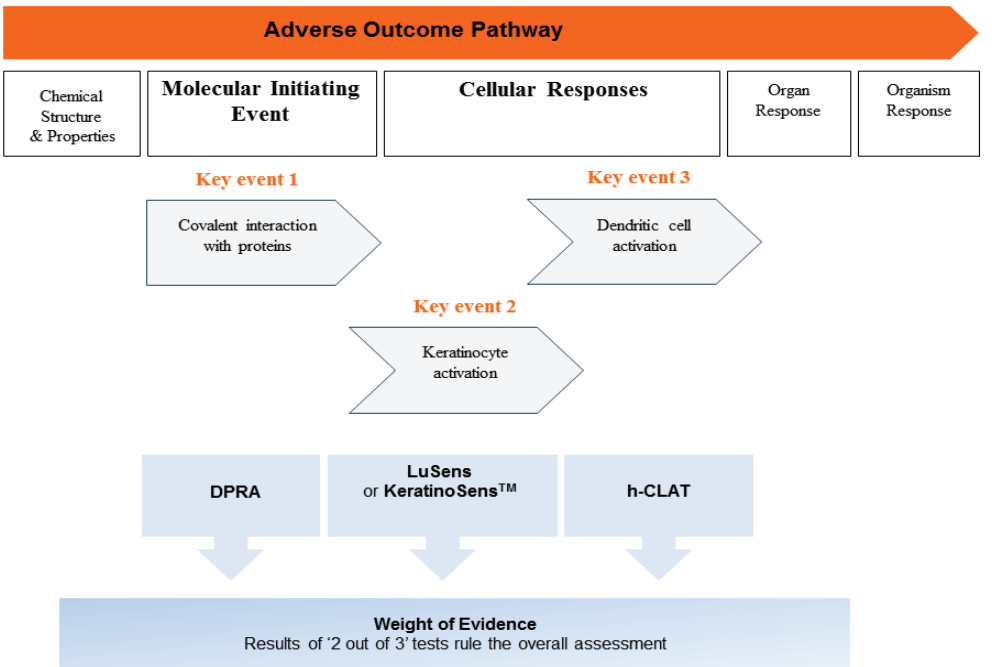


Figure 2.1 *Schematic representation of the AOP for skin sensitization with five non-animal test methods as part of the '2 out of 3' Weight of Evidence (WoE) integrated testing approach addressing the first three key events.*

The non-animal test methods have yet some limitations and may not be amenable for use for substances showing high cytotoxicity, insolubility, volatility, etc.

2.3.4 Integrated Approaches to Testing and Assessment (IATA) Based on Results of Non-Animal Test Methods

To date, OECD TGs are only available for individual non-animal test methods. In contrast, standardised testing strategies using a combination of non-animal test methods are internationally not accepted and thus test guidelines for such testing strategies are not available to date. Currently, the OECD under the lead of JRC EURL is in the process of defining criteria and reporting templates to allow consistent information reporting with the aim to facilitate acceptance of the Integrated Approaches to Testing and Assessment (IATA) for substance evaluations. Reporting of the IATA will need a degree of flexibility as it should be purpose-driven; for example, reporting will depend on whether hazard, risk or safety assessments are made as well as on the regulatory use and the amount of data already being available. Within the IATA, predefined testing strategies (i.e. integrated testing strategy (ITS);

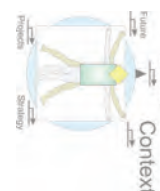
sequential testing strategy (STS)) can be incorporated. A number of strategies were proposed based on results of non-animal test methods addressing individual key events of the AOP. These IATA case studies describe testing strategies used for hazard or potency identification of a test substance by strategically integrating and weighting the relevant data available. This may be performed by a pre-defined procedure (a deterministic approach, similar to traditional and regulatory accepted endpoint methods), a case-by-case evaluation, or pre-defined probabilistic models. According to the current status, eleven different IATA case studies will be available as case studies to be used as reference on how to report the testing approach along with the non-animal test methods (*CEFIC, 2015*).

All eleven IATA case studies (*Table 2.1*) used LLNA and/or human data as *in vivo* reference to determine the performance of the respective underlying prediction model. Most of the input data were obtained from non-animal test methods covering the first three key events, such as the DPRA (OECD TG 442C) and ARE-Nrf2 luciferase test methods, (OECD TG 442D; KeratinoSens™ or LuSens,) as well as the h-CLAT (OECD draft TG). Eight of the approaches also included *in silico* data. Five approaches aim to predict hazard potential and six also provide information on potency. Five models are of deterministic nature and provide a pre-defined and transparent pattern on how to generate an overall prediction. Another five are of probabilistic nature. Four of these probabilistic models are based on machine learning approaches like Bayesian networks, artificial neural networks or support vectors machines.

Table 2.1 *Overview of the 11 IATA case studies.*

Author	AOP key event	*In vivo* reference	Purpose	Type of IATA		Brief description
BASF	1, 2, 3	human; LLNA	hazard	deterministic	majoritiy vote	The '2 out of 3' prediction model provides a deterministic approach using results from three non-animal test methods which can be used to assess skin sensitization hazard potential based on a weight of evidence pertaining to AOP key events including the MIE (*Bauch et al., 2012; Urbisch et al., 2015*).
DuPont	1, 2, 3	LLNA	hazard	deterministic	decision tree	This IATA represents a deterministic approach focusing on relevant existing information (e.g. *in silico*, *in chemico*, *in vitro* and/or *in vivo* data) which can be implemented into a decision tree for assessing skin sensitization potential (*Patlewicz et al., 2014)*.
Givaudan	1, 2	LLNA; (human)	potency	probabilistic	regression model	This IATA represents a probabilistic approach using quantitative concentration-response data from the KeratinoSens™ assay and kinetic reaction rates on peptide reactivity (e.g. Cor1C420-assay) for generating global and local regression models (TIMES SS for defining local domains) to predict LLNA-EC3 or human $DSA_{0.5}$ values (*Natsch et al., 2015*).
ICCVAM	1, 2, 3	LLNA	hazard	probabilistic	machine learning	This IATA combines non-animal test data, physico-chemical parameters as well as *in silico* data (i.e. QSAR Toolbox) in a probabilistic "machine learning" approach to predict hazard potential of a test substance as assessed by the LLNA (*Matheson et al., 2015)*.
Kao #1	1, 3	LLNA	hazard; potency	deterministic	majoritiy vote	This Sequential Testing Strategy (STS) represents a deterministic approach being based on *in chemico* (i.e. DPRA) and *in vitro* data (i.e. h-CLAT) and thus covering AOP key event 1 and 3, respectively, in order to predict skin sensitization potential and potency of a test substance (*Nukada et al., 2013; Takenouchi et al., 2015*).

Kao #2	1, 3	LLNA	hazard; potency	deterministic	score-based battery	The score-based battery system represents a deterministic approach combining *in silico* (i.e. DEREK Nexus), *in chemico* (i.e. DPRA) and *in vitro* data (i.e. h-CLAT) in order to predict skin sensitization potential and potency of a test substance (*Nukada et al., 2013; Takenouchi et al., 2015*).
L'Oreal	1, 2, 3	LLNA	hazard	probabilistic	machine learning	This decision strategy combines results obtained from different *in silico*, *in chemico* and *in vitro* models in a stacking meta model to predict hazard potential with confidence indication (*Teissier & Alépée, 2015*).
P&G	1, 2, 3	LLNA	potency	probabilistic	machine learning	The Bayesian network framework formulates a probabilistic machine learning method for integrating various types of *in silico* (for bioavailability parameters), *in chemico* (i.e. DPRA) and *in vitro* data (i.e. KeratinoSens™, h-CLAT) covering the first three key events of the AOP in order to predict the putative LLNA potency class for a test substance (*Jaworska et al., 2013*).
RIVM	1, 2, 3	LLNA	hazard	probabilistic & deterministic	majoritiy vote	The RIVM STS represents a deterministic approach (with probabilistic elements in tier 1) being constructed as a tiered strategy for the assessment of skin sensitization hazard potential with a decision point at the end of each of the three tiers using *in silico* (various models), *in chemico* (i.e. DPRA) and *in vitro* data (i.e. KeratinoSens™, Gene signature assay, h-CLAT; *van der Veen et al., 2014*).
Shiseido	1, 2, 3	LLNA	hazard; potency	probabilistic	machine learning	This probabilistic IATA describes the prediction of LLNA potency values by combining different *in vitro* (i.e. SH test, AREc32 assay, h-CLAT) and *in silico* parameters in an artificial neural network (ANN) model (*Hirota et al., 2013; 2015; Tsujita-Inoue et al., 2014*).
Unilever	1, 3, 4	human	risk assessment	quantitative modelling		This IATA uses experimental data on dermal penetration and hapten-peptide interactions to quantitatively model human $CD8^+$ T cell response (*MacKay, 2015*).



2.3.5 Conclusions

Most of the traditional *in vivo* toxicity tests (like the GPMT and Buehler test) directly detect the adverse outcome; the LLNA detects the sensitization but not the elicitation phase. Assessing skin sensitization by using non-animal test methods actually presents a novel situation: (i) The full AOP is known; (ii) *in silico*, *in chemico* and *in vitro* methods are available for different events of the AOP; (iii) the individual test methods have been combined into different testing strategies and the prediction models of such testing strategies are being evaluated (in addition to the validation studies for the individual non-animal test methods). All of these issues result in the option to assess skin sensitization with a testing strategy addressing different key events of the AOP – thus, not the adverse outcome itself is assessed.

No single non-animal test method for skin sensitization is currently considered to be sufficient for use as a 'stand-alone' method for hazard assessment and instead integrated approaches to testing and assessment were developed. This is also reflected in the OECD TGs 442C and 442D in which it is stated: 'Therefore, data generated with this TG should be considered in the context of integrated approaches such as IATA, combining them with other complementary information e.g. obtained from *in vitro* assays addressing other key events of the skin sensitization AOP as well as non-testing methods including read-across from chemical analogues'.

The availability of non-animal test methods to analyze different AOP key events of the skin sensitization process is not the limiting factor (albeit the resources and time required for development, validation and translation into OECD TGs may be limiting). Rather, clarification is needed on which data are essential for hazard assessments (particularly for regulatory purposes) and further also for risk assessments.

Skin sensitization is the first example where substantial progress has been made by defining key events of an AOP. Furthermore, OECD TGs for experimental non-animal test methods covering the first three key events have been adopted or are at final draft stage. Even though the data requirements have been defined and sufficient non-animal test methods are available, there is yet still a need for regulatory acceptance of testing strategies such as IATAs to link the individual data into a hazard or risk assessment. This is one of the major lessons to be learned: Hazards from complex toxic effects cannot be assessed by only one non-animal test method and translating the results of a sufficient set of non-animal test methods into a hazard assessment is one of the major challenges. It remains to be seen whether this challenge is best met by predefined deterministic or probabilistic models or entirely on a case by case basis.

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2.4 RISK 21 - A Roadmap for 21st Century Human Health Risk Assessment

Alan Boobis, John Doe, Michelle R. Embry, Timothy P. Pastoor

2.4.1 Background

For almost half a century, human health risk assessment has relied on animal testing to identify and characterise adverse effects of chemicals. Traditional animal testing cannot adequately or efficiently accommodate the thousands of chemicals currently in use that remain to be assessed. In addition, there are societal and ethical demands to reduce the use of animals, and calls for greater clarity and transparency in determining and communicating human safety (*NRC, 2007*). The last decade has seen unprecedented efforts to develop non-animal methods (computational and in vitro) to assess the toxicity of chemicals, underpinned by the concept of toxicity pathways/adverse outcome pathways/modes of action (*Tollefsen et al., 2014*).

To address and catalyse improvements in human health risk assessment, by developing a framework to enable the integration of information obtained by both conventional and emerging methods, the Health and Environmental Sciences Institute (HESI) created the RISK21 Project.

RISK21 is coordinated by HESI, a global branch of the International Life Sciences Institute (ILSI, see also a short characterisation in section 5.2.2), and is a multi-sector program, with participants from government agencies, academia, industry, and others (*www.hesiglobal.org*). The project has engaged over 120 participants from 12 countries, 15 government institutions, 20 universities, and 2 non-government organisations since it was formed in 2010.

2.4.2 RISK21 Principles

RISK21 (*Pastoor et al., 2014*) derived a flexible framework for bringing together knowledge to enable effective, efficient decision-making. It is based on a set of principles:

- Focus on problem formulation
- Utilise existing information to the extent possible
- Start with exposure rather than hazard
- Use a tiered approach to data development and decision making

Focus on problem formulation

Problem formulation is built around the fundamental question: 'What decision do you need to make?' For example, for priority setting, the problem formulation would limit the scope of the assessment to determining which chemicals are of greatest concern. For a full human health risk assessment, the problem formulation would focus on identifying sufficient information on use, exposure, and toxicity to establish a margin of exposure (MoE) and a decision as to whether or not that MoE provides reasonable certainty of no harm. Risk assessments should begin with the end in mind by considering physical/chemical properties, use characteristics, existing exposure and toxicological data, and the risk management context. By starting with these disciplined and transparent steps, there is greater likelihood that appropriate and necessary data are developed without unnecessary commitment of resources.

Utilise existing information to the extent possible

There are few chemicals that are so isolated in their properties, effects, or exposure characteristics that some indication of their potential toxicity, mode of action, or human exposure cannot be estimated from similar chemicals or uses. By collating and mining the extensive knowledge that now exists on chemistry, fate, use characteristics, and toxicity, useful predictions about the behaviour of a chemical can be obtained.

Decades of data generation, much of which is now available in online databases, can be utilised to provide estimates of exposure and toxicity that may be sufficient to make a decision or, if not, to guide focused data generation.

Start with exposure rather than hazard

Human safety depends on both exposure and hazard. An early estimate of potential human exposure in relevant scenarios and populations, including susceptible populations, will characterise the degree of specific toxicological data needs. For example, chemicals with exceedingly low potential exposure should lead to less allocation of toxicological resources than those with higher exposures, which could call for a more extensive toxicological database to inform risk assessment.

Use a tiered approach to data development and decision-making

RISK21 utilises a tiered approach for both exposure and hazard assessment. This leads to the optimal use of available resources and establishes a value of information approach for decision-making. Guided by problem formulation and taking advantage of existing information, both exposure and toxicity estimates can be generated, initially at a basic level, that may be sufficient for a decision about human safety.

Data acquisition can stop when there is enough precision to make a decision. 'Precision' is used here to represent the degree of exactness in the data, which is usually an estimate bounded by a confidence interval or range, the size of which is typically proportional to the quality and quantity of knowledge used to generate the estimate.

Usually, greater investment of resources is rewarded with narrower ranges of estimates, and hence greater precision. In some cases, a broad estimate of exposure or toxicity may be sufficient, while in other cases, more precision will be necessary.

2.4.3 The RISK21 Roadmap

RISK21 organised the above principles into a transparent and tiered framework called the RISK21 roadmap (*Embry et al., 2014*). Estimates of exposure and toxicity are plotted on the RISK21 matrix, with an estimate of their respective bounds of uncertainty, providing a highly visual representation of the margin of exposure. It can easily be ascertained whether there is likely to be a health concern and if so, whether it would be more productive to refine the estimate of exposure or of toxicity (*Figure 2.2*). Use of this matrix allows dynamic illustration of the impact of moving through the respective assessment tiers (exposure and toxicity) and the consequences of reducing uncertainty in the estimates. The axes can be any suitable dose metric, from external exposure to target tissue concentration, as long as it is the same on both. In this way, the matrix can readily accommodate data generated *in vivo*, *in vitro* or using a combination of approaches.

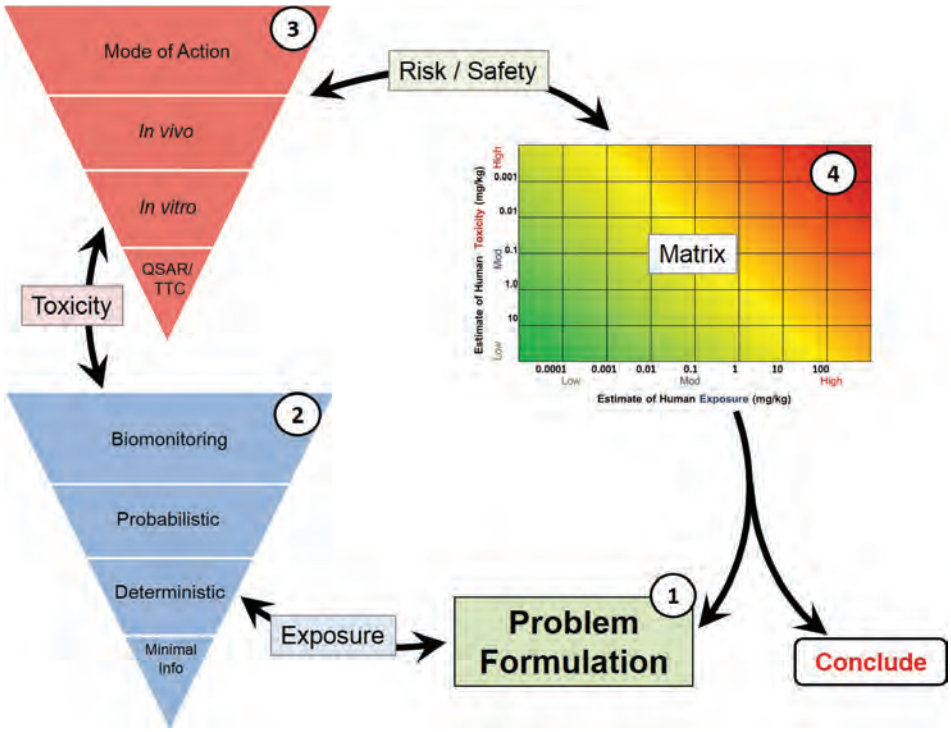

Figure 2.2 *The RISK21 roadmap. This diagram is a schematic representation of a multifunctional tool that provides a transparent process for obtaining rational risk-related decision points. The inverted triangles for exposure and toxicity represent the proportional investment of resources needed for each tier. The following steps describe the use of the roadmap and are described in additional detail in Embry et al. (2014): 1) Problem formulation; 2) Exposure estimate; 3) Toxicity; 4) Matrix (Source: Embry et al., 2014).*

Benefits of RISK21

The current risk assessment paradigm, relying on laboratory animal-based studies, was developed by an arduous process of international negotiation and agreement over decades. It was based on a widely accepted concept that animals should be dosed for periods of time or portions of their life-cycle which mimicked human life (3 months for medium term exposure, 24 months in rodents for lifetime exposure in humans, dosing during pregnancy and neonatal development). The paradigm has been accepted as the 'gold standard' and is the basis of much of the current legislation on chemical safety internationally.

This paradigm is now being viewed as outdated, or at least no longer fit-for-purpose, as it is very resource intensive and therefore unable to cope with the need to assess thousands of chemicals. In addition there is the need to extrapolate results from animals to sensitive

humans, which is generally achieved by using uncertainty factors of 100 or 1000. Although seen as the gold standard, the paradigm has therefore an in-built imprecision of 2-3 orders of magnitude.

New methodology is being developed which aims to address both the issue of the use of resources, especially the use of laboratory animals, and the issue of extrapolation to humans. However it is unlikely that a consensus will emerge on the acceptability of the methodology as easily as there was for the current animal-based paradigm. In fact, the aim is not to replace one fixed set of assays with another fixed set of assays but rather to develop an approach enabling the toxicological profile of a chemical to be characterised. Science and understanding of adverse effects of chemicals will continue to advance in the future and it will be preferable to incorporate new understanding into the assessment methodology as it emerges and becomes accepted; evolution rather than revolution. In this way the static nature of the 20th century testing regime (*Hartung, 2009*) can be avoided whilst at the same time ensuring that there is a realistic prospect of political and regulatory acceptance of new methods.

The key is to understand the precision of the methodology, the degree of exactness of the data in predicting effects on human health. Once this is known the RISK21 road map allows a method to be incorporated into the tiered approach. The approach is non-judgmental with regard to the methodological origin of the data, as long as they can be expressed in a common metric. The greater the precision of the methodology, the higher the tier at which it can be used. However, many decisions about the safety of chemicals in use can be made using lower tier assessments. It is also important to emphasise that the RISK21 approach does not mandate a rigid hierarchical application of the tiers. These can be applied as appropriate, with different tiers for exposure and hazard if necessary and moving to whichever higher tier is most suitable.

The RISK21 approach can provide a framework which can bring together the greater understanding of modes of action, adverse outcome pathways, toxicokinetics, high throughput assays, exposure models, databases, *in silico* algorithms and the other advances which are being made by many groups. As these develop, they need a framework which allows them to work together. We are not trying to develop a new set of assays to replace the current paradigm, we are trying to develop a new framework which will allow advances to be incorporated as they are made. The framework will be constant, but the components within it will evolve as the science develops.

RISK21 provides a transparent framework that incorporates exposure and toxicity estimates and their level of precision to guide informed decision making. It represents a step forward in the goal to introduce new methodologies into 21st century risk assessment.

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2.5 Green Toxicology

Thomas Hartung

2.5.1 Introduction

Alternative methods in toxicology are increasingly enabling technologies, i.e., they can do more than optimise and replace current regulatory testing. Historically, early identification and characterization of adverse effects of industrial chemicals was difficult because conventional toxicological test methods did not meet R&D needs for rapid, relatively inexpensive methods amenable to small amounts of test material. The pharmaceutical field has for some time taken advantage of new *in vitro* and *in silico* technologies for front-loading testing and mechanistic understanding for early determination of possible toxic liabilities. The chemical industry has begun to embrace similar concepts from the Green Chemistry movement. A Green Toxicology is emerging, which uses structure-activity relationships for the design of less harmful substances, tests early in the development process to prioritise less dangerous chemicals, and reduces exposures – thereby reducing risk and testing demands. These approaches promise to create opportunities for the development and use of alternative test methods and will support the transition to sustainable chemistry.

Over the past few decades, there has been an increase in consumer demand for less toxic, more environmentally-friendly products, as well as increasing regulatory and economic pressures for more sustainable products, less wasteful manufacturing, and a switch to renewable resources as source materials – in essence, a *Green Chemistry* approach (*Anastas & Warner, 2005*) that puts environmental and sustainable principles at the forefront of chemical design. The principle of "benign design" has been part of the 12 founding principles of Green Chemistry from its inception (*Anastas & Warner, 1998*), and principles three and four directly address this:

3. *Wherever practicable, synthetic methodologies should be designed to use and generate substances that possess little or no toxicity to human health and the environment.*

4. *Chemical products should be designed to preserve efficacy of function while reducing toxicity.*

Other principles aim to reduce waste and use of chemicals and thus limit exposure in the environment and the workplace.

The current industrial product development paradigm relies on time-consuming, expensive animal studies and is too slow to keep pace with technological change (*Hartung, 2009; 2010*). For example, a typical two-generation reproductive study costs more than $500,000, uses more than 3000 rats, and takes 15 months to complete. For this reason, toxicity testing is typically reserved for the latter stages of chemical/product development (after the chemical or product is determined to be commercially viable). Consequently, toxic effects are identified closer to commercialization when few options for design changes exist and after significant investment of time, resources, and money. Today, rapidly evolving, 21st century safety assessment methodologies have the potential to transform how companies develop and commercialise new products and chemicals

This rapid, high-throughput, high-content *Green Toxicology* paradigm can work in tandem with R&D by providing answers about mechanisms of toxicity quickly, inexpensively, and with the small quantities of material typically available for R&D. *Green Toxicology* combines the *in vitro* and *in silico* tools of predictive toxicology with the principles of chemical design to develop chemicals that have negligible toxicity. It also enables early elimination of candidates possessing undesirable traits by 'failing early and failing cheaply' – or, to put it more positively, innovation through early and inexpensive evaluation of hazard.

2.5.2 Benign Design

The idea is simple: toxicologists partner with synthetic chemists to understand what chemical moiety may impart undesired hazard traits as early as feasible in product development.

Toxicology is in the midst of a major transition from animal-based methods that are slow, expensive, and suffer from low-throughput to more modern approaches utilising cheminformatics, cell cultures, genomics, and computational biology to achieve greater speed and throughput, lower cost, and – ultimately – more accurate predictions for humans and environmental safety (*Leist et al., 2008; Hartung, 2009*). Read-across and QSARs (*Hartung & Hoffmann, 2009*) have helped to make testing more targeted and efficient, their utility in Green Toxicology lies in low cost, rapid 'Tier 1' assessments of new candidate chemistries and sustainable alternatives.

Some positive examples exist, especially in the field of aquatic toxicity (*Voutchkova et al., 2010a; b; 2011*), but this is arguably an easy case, where lipophilicity is key to uptake and thus a hazard. Additionally, while QSARs have certainly proven their merit in the pharmaceutical industry, this success is unlikely, for a variety of reasons, to be repeated for industrial chemicals. Industrial chemicals may consist of polymers with a wide range of molecular weights, various impurities, left over reagents, etc., while the majority of drugs fall into a more narrow chemical space often referred to as the Lipinsky rules (*Lipinsky, 2004*):

- No more than 5 hydrogen bond donors (the total number of nitrogen–hydrogen and oxygen–hydrogen bonds)
- Not more than 10 hydrogen bond acceptors (all nitrogen or oxygen atoms)
- A molecular mass less than 500 daltons
- An octanol-water partition coefficient log P not greater than 5.

This is a nice example (though not for safety but efficacy) of how structure considerations can help substance design.

More fundamentally, QSARs developed for the pharmaceutical industry have a domain defined by suspected biological activity. QSARs may simply lack the accuracy necessary when the overwhelming number of chemicals are, in fact, lacking toxicity, as is the case for many industrial chemicals. Therefore, while QSARS likely will have a role to play in the development of benign alternatives, it is equally important that toxicology develops other techniques and approaches that link molecular structure with toxic outcomes in a way that can be useful to synthetic chemists.

2.5.3 Test Early, Produce Safe

The pharmaceutical industry has developed the concept of *fail early, fail cheap* as a consequence of the cost explosion and high failure rates in late clinical development (*Hartung & Zurlo, 2013; Hartung, 2013*). It was noted that in the 1990s, for example, a large number of drugs failed because of pharmacokinetic problems, i.e., the active agent did not reach

sufficient concentrations in the targeted organ in patients. Addressing this early and with human relevant methods markedly reduced this type of failure (*Singh, 2006; Tsaioun & Jacewicz, 2009*).

This approach can also be adapted for front-loading toxicity testing of industrial chemicals. In the short term, predictive safety assessment offers a way to enrich the R&D pipeline for chemicals that are most likely to clear challenging regulatory hurdles. Because predictive methods focus on the root causes of toxicity at the cellular and molecular levels, they also generate new knowledge to inform the design of safer and more sustainable products. Traditional toxicity cost several million dollars for a product to reach the marketplace. These studies also take a lot of time, in some cases requiring years to complete. The rat cancer bioassay, for example, entails two years of treatment plus time for planning, histopathology, and reporting. And often, at the end of this process, the results are equivocal and may be of questionable relevance for humans (*Basketter et al., 2012*). If the results are positive, such bioassays typically provide no mechanistic information required for a synthetic chemist to design a less toxic alternative. Under the pressure of *time-to-market* and the running clock of the patents and competitive economic pressures, these are clearly not the best tools for early decision making.

Front-loading thus requires screening-level tests that are both less costly and much faster and contribute to a smarter approach that begins with *in silico* screening to predict possible targets and progresses to targeted *in vitro* tests that can examine suspected Pathways of Toxicity (*Hartung & McBride, 2011; Kleensang et al., 2014*). For those candidates that do move on to whole-animal tests, a smarter testing approach might allow for reduced reliance on high-dose testing that causes gross pathological change as an indication of toxicity and focuses more precisely on the molecular initiating event at doses that can meaningfully be related to possible human exposures.

Another advantage of front-loading toxicity in the R&D process would be to reduce replacements that are promoted as alternatives to known 'bad actors' often turn out to be not necessarily less *toxic,* but simply have less *data* (as was the case with flame retardants; *Lakind & Birnbaum, 2010*). This creates a somewhat perverse incentive to avoid gathering toxicity data, which is compounded by the fact that consumer preferences can be markedly influenced by the results of toxicity tests taken out of context. More rigorous toxicity testing as an essential part of the R&D process would likely produce a more rational selection of benign replacements.

2.5.4 Avoid Exposure and Thus Testing Needs

Traditionally, toxicologists are trained to think in terms of molecules and not in terms of the production processes behind them. Within the many steps involved in the production of

industrial chemicals, however, small alterations can often achieve significant reductions in terms of exposure and therefore minimise risks. Toxicity, for example, may reside in a catalytic agent that can be eliminated with alternative routes of synthesis. For many polymers, the final product has a sufficiently large molecular weight so as to preclude bioavailability, and any hazard is likely due to residual monomers. Consequently, small changes in the efficiency of the reaction or the purification step can drastically reduce the hazard while conserving resources. Similarly, a change to *one-pot synthesis* (meaning that all reactions take place in the same vessel) can decrease the number of exposed workers. In this respect, the goals of Green Toxicology dovetail with Green Chemistry's goal of improved efficiency and emphasis on the importance of close collaboration between the chemist and the toxicologist. Together their measures directly affect occupational health and then (via reduced exposure) influence risk assessment and management. Such scenarios are ripe for exposure-driven testing strategies, which can result in reduced testing demands. Reduced exposure also makes it more likely that Thresholds of Toxicological Concern (TTC) are not exceeded (*Kroes et al., 2005; Munro et al., 2008*) – an example of a formalised, exposure-driven assessment.

In conclusion, Green Chemistry – by reducing exposure and thus testing demands – has more to offer to toxicology, safety testing, and risk management than just Benign Design and Early Testing (*Figure 2.3*).

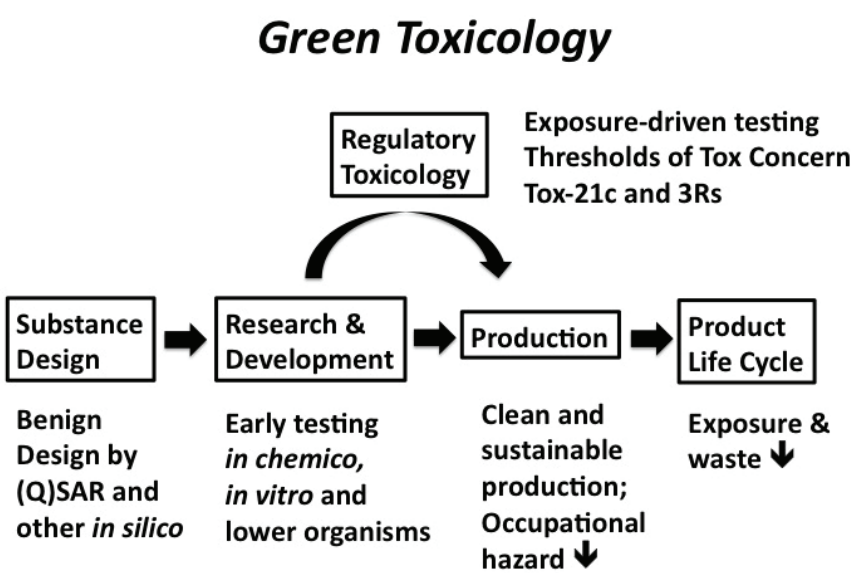

Figure 2.3 *Principles and results of Green Toxicology (source: Maertens et al., 2014; reproduced with permission).*

2.5.5 Make Testing Sustainable

Animal testing is not just costly in terms of time and money, but is inefficient with regards to resources and energy and produces a great deal of biohazard waste. Consequently, we argue that the long-term use of animals is fundamentally unsustainable. It has been estimated that regulatory requirements in Europe require approximately 300 animals to test new chemical compounds and up to 10,000 animals for a pesticide (*Bottini and Hartung, 2010*). The 10,000 animals per pesticide, it should be noted, does not include abandoned products. Before REACH in Europe, 90-140 thousand animals were used for testing roughly 200 new chemicals per year, not including testing outside of Europe. In the US, discrepancies in testing demands are even stronger between different products, with seven out of eight new industrial chemicals having no toxicity data at pre-marketing notification under the Toxic Substance Control Act (*Hartung, 2010*) and similar requests for more than 30 animal tests for pesticides. With 40,000 animals tested for safety per new substance entering the market and 350,000 for R&D (factoring in the animals used for candidate drugs that do not succeed), the pharmaceutical sector still uses a large number of animals despite the impressive reductions in recent years. This is not only unsustainable but may impose an economic barrier that is prohibitive for niche chemicals with limited profitability. A smarter, *in vitro* testing strategy can reduce the use of resources for testing by better prioritization and more efficient screening-level tests. In the longer term, we hope agencies will find greater application of predictive methods to address some of the requirements of their programs.

2.5.6 Early Testing Can Use Methods not Yet Mature Enough for Regulating

Regulation tends to take a precautionary approach oriented towards minimising mistakes rather than optimising the cost/benefit analysis, making it profoundly difficult to change traditional approaches. Furthermore, a traditional validation study takes about one decade. Consequently, a validated test is 'frozen in time', and it is simply impossible for regulatory mechanisms to keep up-to-date with the current rate of change in science (*Hartung, 2007; Leist et al., 2012*).

Frontloading toxicity at the research and development stage, however, allows a more flexible approach. Prioritization of substances for development can be based on methods, which still have some margins of error. Early testing allows the use of methods not yet validated. *In silico* and *in vitro* tests that are individually too inaccurate for regulatory purposes will likely have a useful place in an integrated testing strategy (*Hartung et al., 2013; Leist et al., 2014; Rovida et al., 2015*). Such strategies allow uncertainty in results and seek to combine data from multiple tests in a flexible manner that maximises predictive power while also providing an estimate of the uncertainty in the data. This also helps to build capacity and capability to perform these assays for later regulatory use, if validated and accepted. In the meantime, front-loaded

methods will be generating data, and thereby facilitating an assessment of the predictive value of these methods and thus contributing to the validation and acceptance process.

This also opens up a role for new risk assessments based on 'toxicity pathways' (cell/molecular level changes) and data-driven uncertainty factors (e.g., intra-human variability factors based on genetic analysis). It will take tremendous time to base regulatory testing on toxicity pathways / Adverse Outcome Pathways (AOP), as the respective database would first need to be sufficiently and comprehensive and validated (*Hartung & McBride, 2011*). The Human Toxome project has started exactly this (*Bouhifd et al., 2015*). With each and every toxicity pathway identified, however, the respective assays can be included in integrated testing strategies. A pathway-based approach can also allow for more precise understanding of individual variation in response to toxicity as well as susceptible populations by illuminating more precisely the differences in toxicity pathways.

Green toxicology can serve as a bridge between 21st century toxicology methods and the development of safer, sustainable products. This paradigm shift and transformation is necessarily a slow and lengthy process as the safety of workers and consumers is at stake. This delay makes regulatory science less attractive for academic research and even less so for the commercialization of test methods. If companies have to wait a decade for the regulatory acceptance of a test with unclear prospects for the validation phase, the return on investment is rather unlikely. Early non-regulatory testing creates an immediate market for new test methods, liberating the market forces necessary to standardise and disseminate tests internationally.

Acknowledgement

This article is based on *Maertens et al. (2014)*.

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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, Tilman Gocht

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*). Volume 4 provided detailed descriptions of the **SEURAT-1** proof-of-concept case studies (*Berggren et al., 2014*). This volume discusses the concrete work on proving **SEURAT-1** concepts that is currently 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 SEURAT vision is to fundamentally change the way we assess the safety of chemicals, by superseding traditional animal experiments with a predictive toxicology that is based on a comprehensive understanding of how chemicals can cause adverse effects in humans (*Whelan & Schwarz, 2011*). 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. One of the **SEURAT-1** objectives is to demonstrate a multiple level proof-of-concept (theoretical, methodological, and application; *Whelan & Schwarz, 2012; 2013*).

The multiple proof-of-concepts are based on the idea that the application of a toxicological mode-of-action framework for chemicals safety assessment can be split into three steps (*Figure 3.1*). Firstly, development of a theoretical Adverse Outcome Pathway (AOP) describing the key events of the biological process initiated by a chemical stressor (*Boobis et al., 2008; Ankley et al., 2010*), if possible including quantitative considerations, i.e. resulting in a quantitative AOP

(qAOP). Secondly a testing strategy is needed for toxicity prediction, based on (q)AOP(s) knowledge relevant to the toxicity to be predicted. Typically more than one test method would be needed to trigger selected key events, in combination with *in silico* profilers or *in chemico* predictors. Finally, the results of testing strategies, in combination with already existing data (such as physical chemical information, animal or human *in vivo* data) and kinetic modelling, could provide sufficient evidence to support chemical safety assessment. In the last and third step careful consideration should be made to include the reasoning as outlined in the conceptual framework developed within **SEURAT-1** (see section 4.10.9).

Figure 3.1 *The three levels of the* ***SEURAT-1*** *proof-of-concept case studies.*

Many of the **SEURAT-1** activities are connected to target organ systemic toxicity after repeated exposure to liver. Theoretical AOP constructs were drafted for fibrosis, steatosis and cholestasis (*Landesmann & Vinken, 2013*). A level 1 case study on drug-induced cholestasis was conducted for further elucidating this pathway. The case study was described in the previous volume of the **SEURAT-1** Annual Report (*Berggren et al., 2014*) and the first results demonstrating predicted key events are described here in section 3.3.1 (see below).

At the 5th **SEURAT-1** Annual Meeting (held in Barcelona in January 2015) current results from the seven level 2 case studies were presented. They are still to be finalised and the results at the time of preparation of this Annual Report are briefly summarised in sections 3.4.1–3.4.7.

The prediction goals of the seven level 2 studies are (*Berggren et al., 2014*):

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. Molecular Initial Events (MIEs) associated to liver steatosis
7. Liver/non-liver toxicity.

The chemicals selected to be tested in the case studies are used for benchmarking, i.e. they are chosen to test the predictive system, based on their already known modes-of-action and related adverse effects. **SEURAT-1** standard reference compounds (*Toxbank, 2014*; *Wiseman, 2012*) relevant for target and predictive toxicity have been included in the test systems. In a few case studies it was necessary to select additional substances. The chemical selection was then carefully described to enable interpretation of the results.

The results from the predictive toxicity testing at the level 2 proof-of-concept, besides having a value in themselves, will also be used in the level 3 case studies. It was agreed that the consortium would carry out two separate case studies for applied safety assessment: the *ab initio* and the read-across case study (*Berggren et al., 2014*; see also sections 3.5.1 and 3.5.2 below). The *ab initio* case study will use data from the **SEURAT-1** methods to make a risk assessment for repeated dose toxicity and predict a no-effect level of a cosmetic ingredient assuming a certain exposure scenario. The primary goal of the read-across case study is to increase confidence in read-across assessment by using data from alternative methods. This approach will use a no-effect level based on existing data for one substance (the source) and read it across to a similar substance (the target), and the resulting safety assessment is expected to reach regulatory acceptable standards within the **SEURAT-1** timeframe. In contrast, the *ab initio* case study, which solely relies on data from alternative methods, is considered an initial step towards a new alternative risk assessment strategy. The Joint Research Centre (JRC) organised a workshop in Ispra, Italy, 29-30 April 2014, entitled 'The read-across case study for safety assessment contributing to the **SEURAT-1** proof-of-concept' (*Berggren et al, 2015*). Invited experts defined the read-across case study and the outcome of the workshop was the basis for the further work led by Terry Schultz. In a first phase it was agreed to develop a common read-across criteria and templates for comparison of the different read-across exercises and evaluation the robustness/confidence in read-across assessment. This first phase was already finalised (*Schultz et al., 2015*). The planning of the *ab initio* case study was also kicked off in a workshop organised by the JRC in Ispra, 9-10 October 2014, and the outcome of the meeting and the strategy for the *ab initio* case study is briefly described in section 3.5.2.

In addition to those two level 3 case studies, the COSMOS project worked on a model to predict a dermal Threshold of Toxicological Concern (TTC), which was decided to be included as a third and complementary case study on proof-of-concept level 3 (see section 3.5.3 below).

The three level 3 case studies are all based on the conceptual framework (see section 4.10.9), and are complementary to each other (*Figure 3.2*). The TTC, being the simplest model,

assumes that there are no specific concerns based on current exposure and knowledge and that a concentration cut-off value is protective enough. The read-across case study is based on the idea that regulatory accepted data for a source substance can be applied to a target substance, by showing chemical and biological similarity between the source and target substance. In the *ab initio* case study there is no data available for any other chemical similar enough to the one being assessed, neither is there enough information to exclude major concerns and apply a TTC value. This case study, where the safety assessment is only based on alternative data, is the most challenging. However, a structured argument for how an *ab initio* assessment can be developed will be presented under the level 3 proof-of-concept. It is expected that this case study will not reach regulatory acceptance within the time frame of **SEURAT-1**. However, it is a first attempt to confront such a situation and identify data needs.

Figure 3.2 *Approaches of the three level-3* ***SEURAT-1*** *proof-of-concept case studies.*

The TTC assessment is based on already existing information, while for the read-across assessment supporting evidence from alternative methods will support regulatory acceptance. For an *ab initio* quantitative risk assessment, a well-defined alternative integrated testing and assessment strategy is required. The final results from the **SEURAT-1** case studies with emphasis of the level 3 case studies will be presented at the final **SEURAT-1** symposium planned to take place in Brussels, 4 December 2015.

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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, Robim M. Rodrigues, Vera Rogiers

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 liver-based *in vitro* models were selected: 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). These three models were exposed to bosentan, a prototypical and potent cholestasis-inducing drug selected from the ToxBank Gold Compound list. The focus of this case study is the characterisation of established and new biomarkers of cholestatic insults. 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 are identified by applying a number of '-omics'-based technologies, namely transcriptomics, epigenomics, proteomics and metabonomics. These new biomarkers are used as the basis for inclusion of as yet unidentified key events in the AOP.

In a preliminary set of experiments, an appropriate concentration range of bosentan was established. In particular, three concentrations were tested, namely an IC_{10} concentration based on the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-dihphenyl-2H-tetrazolium bromide (MTT) test as well as 25% and 10% of this concentration. These concentrations were established separately in the three liver-based *in vitro* models. In a subsequent series of experiments, the liver-based *in vitro* models were 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 were exposed for another three days to bosentan-free culture medium. At the three indicated times, samples were taken for further testing. Microarray analysis was performed to establish 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. A reporter gene assay for nuclear receptor activation has been developed. For identifying new biomarkers of drug-induced cholestasis, transcriptomics, proteomics, epigenomics and metabonomics have been used.

Preliminary MTT-based establishment of working concentrations of bosentan in HepaRG cells and hSKP-HPCs shows an IC_{10} concentration of 250 μM and 800 μM, respectively. In a first round of experiments, HepaRG cells and hSKP-HPCs were exposed to their corresponding IC_{10} concentrations for 24 hours, followed by sampling for transcriptomics analysis. As such, more transcriptional changes were observed in hSKP-HPCs than in HepaRG cells. However, for the latter, these changes are much more in compliance with the AOP compared to hSKP-HPCs. Furthermore, concomitant experiments to characterise the presence and activity of the bile salt export pump in hSKP-HPCs and HepaRG cells show no significant functional expression in the former. Therefore, further focus was only put on HepaRG cells in this case study. Besides confirming existing information blocks, the first experiment in HepaRG cells also indicated other important transcriptional events, which can be considered as potentially new key events and thus biomarkers. In a second experiment, HepaRG cells were exposed to 250 μM, 62.5 μM (*i.e.* 25% of IC_{10} concentration) and 25 μM (*i.e.* 10% of IC_{10} concentration) of bosentan for 1 and 24 hours as well as after wash-out, followed by sampling for transcriptomics and metabonomics analyses. The results of the metabonomics analysis suggested a critical role for mitochondrial dysfunction in bosentan-induced liver toxicity. In a third and currently ongoing round of experiments, HepaRG cells are exposed to 250 μM of bosentan for 1 and 24 hours as well as after wash-out, followed by sampling for proteomics and epigenomics analyses.

The preliminary results confirmed several key events of the AOP by using HepaRG cells as a liver-based *in vitro* model and bosentan as a prototypical cholestasis-inducing drug. Furthermore, a number of potentially new transcriptomics-based and metabonomics-based key events have been identified. This is now being complimented at the proteomic and epigenomic level. In parallel, several reporter gene constructs for key events in the AOP are currently generated and will be used to identify possible cholestasis-inducing compounds. Furthermore, all experiments will be repeated using PHHs in order to check the robustness of the AOP and the findings of this case study.

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 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 were tested in the *in vitro* setup and relevant biomarkers indicative of liver fibrosis were monitored.

The approach can be divided into three main phases. In the first phase, a cell culture setup was optimised in order to have functional hepatocytes and HSCs for 21 days. This optimisation was done in 3D Hepatocyte/HSC spheroid co-cultures of human hepatic cells kept in 96-well plates. Acetaminophen (APAP) was used as a proof-of-concept compound. APAP hepatotoxicity could lead to HSC activation within 24 hours as evidenced by gene and protein level changes in profibrotic markers. Meanwhile, the model was further characterised as a good *in vitro* model to test liver fibrosis and we further challenged it with reference fibrotic compounds.

Besides APAP, the 3D HepaRG/HSC co-cultures were exposed in the second phase to methotrexate (shown to induce fibrosis in some human subjects after prolonged treatment) and allyl alcohol (often used to induce fibrosis in rats) both in a single- and repeated-dose fashion. Due to the need to constantly expose the cells to the compounds, culture conditions were adapted to include serum-free medium (in collaboration with Fozia Noor and Elmar Heinzle from the NOTOX project). At the end of the 21 days, we observed that cell toxicity was different in single and repeated dose for each compound, but also with different dose-dependent profiles depending on the compound. Furthermore, HSC activation could also be detected especially in repeated exposure, however, not always correlating with the compound toxicity levels. At this stage we have shown that 3D HepaRG/HSC organoids have the capacity to respond positively to the reference fibrotic compounds particularly in a repeated dose scenario that is reminiscent of the development of human fibrosis. Furthermore, the system also demonstrates different responses depending on the nature of the compound.

For practical reasons, both these phases were 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.

3.4.2 Evaluation of Valproic Acid Induced Steatosis in HepaRG Cells

Fozia Noor, Elmar Heinzle

This case study investigated the repeated-dose toxicity mediated via oxidation of fatty acids and leading to increased accumulation of lipids in vesicles. 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 (VPA), which is known to cause hepatotoxicity via steatosis and is contained in the standard reference compound list prepared by Toxbank (*Jennings et al., 2014*). VPA 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*). VPA is also known as an HDAC inhibitor. Detailed molecular mechanisms of action are, however, still poorly understood. Already in the last **SEURAT-1** Annual Report (*Noor et al., 2014*) we reported promising results for toxicity assessment in 2D cultures with extrapolation to oral equivalent doses (OED) using the method of *Wetmore et al.* (*2012*).

Within NOTOX we studied effects of VPA and its metabolites 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 was carried out (as described in more detail in the NOTOX section 4.6.3 of this book). The exposed cell cultures were also characterised using electron microscopy. The time-series data provided were already used for setting up a version of mathematical model predicting 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 within NOTOX.

In joint NOTOX experiments we used a special long-term cultivation medium for HepaRG cells suitable for extended '-omics' analyses even in the physiologically meaningful concentration range applied (*Klein et al., 2013*). Clear effects could be observed on the metabolic, transcriptomic and proteomic levels. Epigenetic changes occur mainly on histone acetylation rather than on methylation. A physiological mathematical model primarily based on literature knowledge was already built and incorporated into a human-scale physiologically-based pharmacokinetic (PBPK) model. This will be further improved based on extended bioinformatics analyses still on-going in NOTOX.

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

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3.4.3 Development of Biomarker Based on a Read-Across Use Case on Valproic Acid Analogues

Sylvia Escher, Jan Hengstler

Human safety evaluation in chemical risk assessment is currently mainly based on *in vivo* animal studies. Repeated dose toxicity studies, ranging from sub-acute to chronic exposure, are in use to assess the risk to human health of a given compound. Along with the restriction of animal testing (e.g. for cosmetics) there is an increasing interest in advancing the integration of animal-free alternative methods to eventually reduce or replace animal testing. The aims of the FP7 project DETECTIVE are to gain mechanistic knowledge, define adverse outcome pathways and identify biomarkers of toxicity by combinations of '-omics' technologies. Before

being applied in safety assessment, the so-far identified biomarkers, reflecting key and/or intermediate events of a given AOP, still have to be validated. We describe here the use of a read-across case study to contribute to the validation procedure of predictive biomarkers.

The biomarkers were identified using transcriptomics data from the TG-Gates database. In this database, valproic acid (VPA) was one of the most potent inducers of changes in genes involved in lipid and energy metabolism. Furthermore, a causal relationship could be drawn to apical endpoints in repeated dose toxicity studies with oral exposure, in which VPA induced histopathological alterations such as lipidosis, fatty acid degeneration or vacuolisation of hepatocytes. To test the aforementioned genes involved in lipid and energy metabolism as possible biomarkers of VPA-like hepatotoxicity, ten structurally similar compounds to VPA (consisting of branched and un-branched carboxylic acids) were identified, for which repeated dose toxicity studies were available. The databases from RepDose, IMI eTOX, ECHA CHEM, COSMOS, Leadscope and NEDO were searched as well as peer-reviewed publications in Web of Science and Toxline. Four VPA analogues induced alterations such as fatty degeneration in animal (rat) experiments and were therefore classified as '*in vivo* positive'. Five compounds did not cause any adverse effects in the livers of rats up to the highest tested doses and were classified as '*in vivo* negative'. One compound was defined as 'borderline', because it showed equivocal evidence of deregulated lipid metabolism.

Biomarker testing by quantitative RT-PCR is still under investigation. Different cell types are studied *in vitro*, namely primary human and rat hepatocytes, as well as HepG2 cells. Initial promising results were obtained i.e. the biomarker G6PD (Glucose-6-phosphatase-catalytic subunit, function 'Glycogenolysis and gluconeogenesis') differentiated very well between the *in vivo* positive compound 2-Ethylhexanoic acid and the *in vivo* negative compound 2-Ethylbutyric acid in primary human/rodent hepatocytes. Specific aspects like species differences or deregulation of biomarker at high doses will be evaluated in more detail in the coming months.

Based on these results a concept will be developed to integrate the resulting biomarker data qualitatively in the context of a read-across approach and/or quantitatively into risk assessment and will be compared to traditional risk assessment. Results will be presented at the EUROTOX 2015 conference in Porto.

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

Agapios Sachinidis, Jan Hengstler

In 2014 our research was focused on identification of pathways, which are generally perturbed in liver after acute compound exposure at 2 hours, 8 hours or 24 hours. Three compound concentrations (corresponding to low, middle and high doses) have been analysed *in vitro* in

human primary hepatocytes. The highest concentration tested was close to the cytotoxic range. Analysis has also been performed utilising publically available toxicogenomics databases. Statistically significant data have been extracted for 151 compounds. It has been revealed that the number of modulated genes is proportional to exposure time and the compound concentrations. Accordingly, the highest number of deregulated genes has been revealed at the highest tested compound concentration after 24 hours of exposure. We have shown that approximately one third of the compounds were responsible for the strongest effects while a large fraction of compounds had a weak effect independent of the time and concentration. In follow-up studies we mainly analysed a number of up-regulated genes per compound at highest dose and at 24 hours. Only 32 of the analysed compounds were responsible for the strongest (100-fold) up-regulations within the transcriptome.

Based on initial data analysis we have established a selection value concept for the gene expression responses. Accordingly, only genes that were at least threefold deregulated were considered. Additionally, we have introduced SV20 parameter representing genes, which were significantly modulated by at least 20 compounds. Based on this criterion, all deregulated genes could be separated as providing stereotypic and more compound-specific gene expression responses. Therefore, stereotypic genes could be more relevant when selecting for liver toxicity biomarkers.

A pathway analysis revealed that up-regulated SV20 genes were commonly associated with metabolism, development, protein degradation, stress response and energy metabolism as well as with few other biological functions. Down-regulated SV20 genes were associated with cell-cycle control, DNA synthesis and repair and immune response as well as with few other biological functions. An interesting observation was that a more broad transcriptomics analysis revealed a significant number of SV20 genes that were strongly modulated, including in liver samples from patients with non-alcoholic steatosis, hepatocellular carcinoma and other liver diseases. Therefore, a range of the biomarkers that were revealed based on the current study of hepatotoxicity could also be potentially applied to the detection of liver diseases. Furthermore, a comparison of SV20 genes with genes modulated in human cardiomyocytes under exposure with cardiotoxicants has revealed both tissue-specific and generic transcriptomics responses.

3.4.5 Developing Chemotypes for Mitochondrial Toxicity

Mark Nelms, Steven Enoch, Andrea Richarz, Chihae Yang, Mark T.D. Cronin

Mitochondrial toxicity has been implicated in a range of adverse outcomes with organs such as liver, kidney and heart being the most susceptible. Mitochondrial toxicity can be induced via various mechanisms of action, for example uncoupling of oxidative phosphorylation and inhibition of the electron transport chain. Investigation of these mechanisms may provide

information regarding the Molecular Initiating Event (MIE) for various organ level toxicities induced via mitochondrial toxicity. The aim of this study was to develop an *in silico* profiler, comprised of chemotypes, to identify compounds with the ability to induce mitochondrial toxicity.

Two datasets were considered: i) 288 drug or drug-like compounds extracted from *Zhang et al.* (*2009*), consisting of a variety of chemical classes including phenothiazines, local anaesthetics and carbazoles, of which 171 had been identified as being toxic to mitochondria, ii) 93 hair dye compounds with oral repeat dose toxicity data retrieved from Scientific Committee on Consumer Safety (SCCS) opinions (in collaboration with Profs V. Rogiers and M. Vinken and G. Ates from the Vrije Universiteit Brussel).

Structural similarity analysis was undertaken on compounds in the hair dye dataset, using the Toxmatch software (v1.07). The aim was to group similar compounds and determine initial categories. Categories containing mitochondrial toxicants were investigated further. A detailed literature search was undertaken to elucidate mechanistic information of MIEs leading to the disruption of mitochondria and this information was used in the development of structural alerts. An *in silico* profiler comprising four mechanism-based structural alerts was developed (*Nelms et al, 2015*). The *Zhang et al.* (*2009*) dataset was searched using the ChemoTyper software (https://chemotyper.org/; developed by Molecular Networks) for structural fragments using the Toxprint library of molecular fragments (https://toxprint.org/; developed by Altamira). Chemotypes associated with mitochondrial toxicity were identified by investigating the ability of the fragments to distinguish between mitochondrial toxicants and non-toxicants. Fragments were considered to be associated with toxicity if 80% or more of chemicals containing the fragment were toxic. At least three chemicals had to be identified as containing the fragment. In total within this case study, 30 structural alerts have been developed for mitochondrial toxicity. It was possible to postulate a mechanism for twelve structural alerts, such as the inhibition of the electron transport chain and uncoupling of oxidative phosphorylation via electron and proton cycling. Examples are shown in *Figure 3.3*.

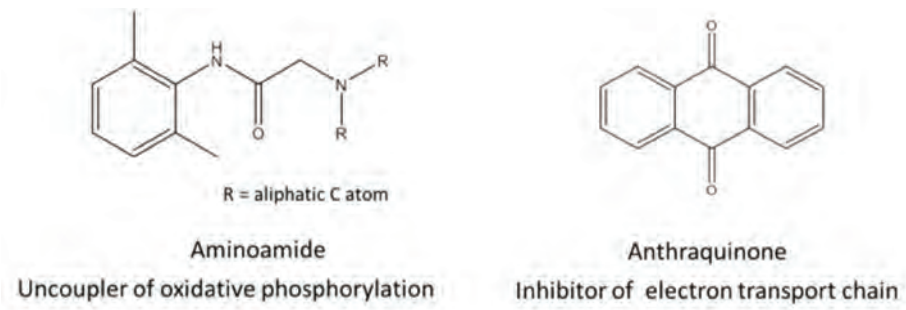

Figure 3.3 *Examples of structural alerts for mitochondrial toxicity with definable mechanisms of action.*

In conclusion, structural alerts were formed by investigating data for organ level toxicity supported by a knowledge of mechanisms and AOPs. The resulting *in silico* profilers can be used to screen datasets for prioritisation purposes and in the development of chemical categories for read-across. For this purpose the structural alerts were coded as SMARTS patterns for convenient use in KNIME screening and categorisation workflows.

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3.4.6 Use of Molecular Modelling Approaches to Predict Potential Binding to Nuclear Receptors Involved in the Development of Liver Steatosis (i.e., LXR and PPARγ)

Elena Fioravanzo, Arianna Bassan, Simona Kovarich, Ivanka Tsakovska, Ilza Pajeva, Anna Palczewska, Vessela Vitcheva, Mark T.D. Cronin, Chihae Yang, Andrew Worth

This case study is a proof-of-concept that molecular modelling (MM) methodologies can be employed in predictive toxicology as part of an integrated strategy. MM methodologies are used here to address specific molecular initiating events (MIEs) involved in the development of liver steatosis (*Landesmann et al., 2012; Al Sharif et al., 2014*): i) the binding to Liver X Receptor (LXR) and ii) the binding to or activation of Peroxisome Proliferator-Activated Receptor γ (PPARγ).

Available crystal structures of human LXR and PPARγ complexes were first extracted from the Protein Data Bank (PDB, http://www.rcsb.org) and experimental data on LXR and PPARγ binding affinity and activation were collected from literature and available databases (e.g., ChEMBL). The ligand-binding domain was explored in detail for each nuclear receptor together with the essential features leading to LXR or PPARγ binding (*Kovarich et al., 2013*). Based on these preliminary analyses, different MM procedures were employed. To predict LXR binding potential, several MM methodologies were used, including both ligand- and structure-based methods (i.e., ensemble docking, e-Pharmacophore and fingerprints-based similarity).

The outcome of the single MM approaches was compared and combined by means of data fusion and consensus modelling. To predict PPARγ binding, a virtual screening protocol was assembled by first applying molecular docking, and then by filtering the generated poses with a pharmacophore model which was generated based on the X-ray complexes of the three most active PPARγ full agonists (*Tsakovska et al., 2014*). 3D QSAR (CoMSIA) models were also developed to predict PPARγ activation.

The procedures developed to predict LXR ligands or PPARγ ligands were then employed to screen two datasets of potential hepatotoxic chemicals, namely a dataset extracted from the COSMOS DB (http://cosmosdb.cosmostox.eu) and a set of molecules collected by JRC for the **SEURAT-1** level 2 classification model for liver toxicity case study (*Lostia 2014;* see also following section). The results provide the basis for both prioritising potential compounds of major concern (for liver toxicity) and/or grouping chemicals potentially sharing a specific Adverse Outcome Pathway (AOP; *Mostrag-Szlichtyng et al., 2014*).

In conclusion, the use of MM approaches to predict receptor binding/activation provides hints for the characterisation of molecular mechanisms that trigger further downstream events and promote the development of liver toxicity. In the AOP framework, MM may thus play an important role by working in synergy with other *in silico* (QSAR, chemotypes, alerts) and *in vitro* approaches. In this case, the *in silico* approach will be further complemented by *in vitro* data on liver steatosis/liver toxicity generated in the context of the JRC level 2 case study to mine existing links between binding to a specific nuclear receptor and liver steatosis (see the following section).

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

Alfonso Lostia

The objective of this case study is to develop and compare the performances of several mode-of-action (MoA)-based classification models aimed at distinguishing between hepatotoxicants and non-hepatotoxicants, with hepatotoxicity defined to cover the three major liver adverse outcomes 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 adverse outcome (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 the hazard profiling of large chemical sets and priority setting (for further testing).

Within **SEURAT-1**, a well-characterised metabolically competent *in vitro* liver system, HepaRG, is exposed to 90 selected reference compounds (75% known hepatotoxicants and 25% known non-hepatotoxicants) and the knowledge of the key events of the three MoAs was used to select the *in vitro* endpoints to be measured.

The selection of reference chemicals is based on clear and robust evidence that they cause or do not cause hepatotoxicity based on animal and/or human data. For this reason, pharmaceuticals were considered as the primary source for data-rich chemicals with high reliability on hepatotoxicity. The reference chemicals are selected to cover the cosmetic structural space and also to have a high structural diversity. To this extent, along with cosmetic chemicals and pharmaceuticals, pesticides and environmental chemicals are also included in the dataset. Furthermore, chemicals already tested in ToxCast and/or TG-GATEs programmes are included to facilitate further data analysis and comparison.

High-throughput screening (using a 96-well plate format) is employed to test the reference chemicals in triplicates and at eleven concentrations each (using a 1:3 dilution factor).

Depending on solubility, the highest concentration to which HepaRGs are exposed is 250 μM in cell medium; all chemicals' concentrations have been tested for solubility by nephelometry analysis. High Content Screening (HCS) assay, based on automated imaging, is used to measure the following *in vitro* endpoints at 4, 24 and 72 hours: cell viability, oxidative stress, mitochondrial damage, apoptosis and accumulation of neutral lipids.

The exposure scheme, designed to be relevant for the prediction of repeated dose liver toxicity, consists of exposing HepaRGs to chemicals every six hours (by replacing the cell medium containing a chemical at a certain concentration) for the overall exposure period of 24 and 72 hrs. The rationale of this protocol is to achieve a relatively constant *in vitro* concentration of a chemical over time. A constant concentration ensures that the observed *in vitro* effects are mainly related to dynamics and less to kinetics (steady-state conditions). The decision of every 6 hours exposure as the frequency to achieve an *in vitro* continuous exposure was based on the comparison of the structural space of the 90 chemicals tested with that of 237 ToxCast chemicals assessed for the *in vitro* metabolic clearance by using primary human hepatocytes (*Wetmore et al., 2012*). The assumption that a continuous exposure is achieved by exposing cells every six hours was made based on the similarity of the two structural spaces and that the majority of ToxCast chemicals had a half-life of less than this time.

To better qualify the toxicity results in the context of the *in vitro* continuous exposure, for the majority of 90 chemicals the concentration of the parent compound will be measured in the intracellular and cell medium fractions after exposing HepaRGs to a chemical, at 1 and 10 μM, and for five incubation times up to six hours. These kinetic measurements can also support the *in vitro* to *in vivo* extrapolation by using available PBPK models, developed within **SEURAT-1**, for selected chemicals.

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).

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

3.5.1 Read-Across Case Study Programme

Terry Schultz, Andrea Richarz

The level 3 read-across case studies are part of the **SEURAT-1** proof-of-concept in the context of safety assessment. The case study scenarios were further shaped during the **SEURAT-1** experts' workshop on 'The read-across case study for safety assessment contributing to the **SEURAT-1** Proof-of-Concept' held on 29-30 April 2014 in Ispra, Italy (*Berggren et al., 2015*). The two-stage program includes the design of a general strategy for developing and reporting a read-across evaluation and the development of four case study scenarios. These are designed to assess each of the most relevant read-across situations:

1. Chemical similarity of compounds that do not require (or do not undergo) metabolism to exert a potential adverse human health effect;

2. Chemical similarity involving metabolism (resulting in exposure to the same/similar toxicant);

3. Chemical similarity with general low or no toxicity;

4. Distinguishing chemicals in a structurally similar category with different toxicological premises.

Stage 1 work culminated with the production of a manuscript (*Schultz et al., 2015*). This document examined category formation, grouping and read-across methods as applied in toxicological assessments and used to fill data gaps for chemical safety assessment and regulatory decisions. Briefly, a transparent and systematic strategy is proposed for evaluating chemical category membership and to support the use of read-across predictions to fill data gaps. The two major aspects of any read-across exercise, namely similarity and uncertainty, are assessed. It is argued that while there can be an over-arching rationale for grouping organic substances together based on molecular structure and chemical properties, these similarities alone are generally not sufficient to justify a read-across prediction, especially for chronic health effect. Typically in such situations further scientific justification is required to give explanation for the chemical grouping. Such justification should include considerations of bioavailability, metabolism and biological/mechanistic plausibility. Uncertainty includes a variety of factors which are by and large divided into two concerns: firstly, uncertainty associated with the similarity justification and secondly, uncertainty associated with the completeness of the read-across argument. Particular to this strategy are templates for guiding the read-across assessment. These templates act to assist in assessing similarity in the context of chemistry,

toxicokinetics and toxicodynamics, as well as to guide the systematic characterisation of uncertainty both in the context of the similarity rationale, the read-across data and the overall approach and conclusions. Ultimately, a workflow for reporting a read-across prediction is recommended.

The strategy, the workflow and the templates are being applied in stage 2 of the work according to the four read-across case study scenarios. The approach consists of a 'traditional' read-across exercise, followed by a second iteration taking into account 'new methods' information (e. g., data from **SEURAT-1** or ToxCast), to evaluate their contributions to reduce uncertainty.

One example of a category of direct-acting toxicant with no metabolism are perfluoroalkyl acids (PFAAs). In this first of the read-across case study scenarios the chemical category is initially based on chemical similarity, i.e., compounds: i) belonging to a common chemical class and sub-classes (perfluorinated, aliphatic carboxylic acids); ii) possessing a common molecular scaffolding (straight-chain C-atom backbone of C_6 - C_{12}); and iii) having common constituents in the form of a single key substituent (-CO_2H), limited structural fragments (-CF_3 and -CF_2-) and a single extended structural group (-CF_2CO_2H). The category is supported by biological similarities in the form of no significant toxicokinetics or metabolism, and the common mechanistic premise of peroxisome proliferator-activated receptor (PPAR) pathways leading to liver toxicity. Specifically, oral repeatedly dosed rodents exhibit liver toxicity, typically in the form of hepatocyte necrosis and increased liver weight due to hepatic steatosis. Briefly, *in vivo* hepatic steatosis is induced via perturbations to fatty acid uptake, lipogenesis, and fatty acid oxidation. The length of the fluorocarbon-backbone appears to impact potency with potency exhibiting a bell-shaped curve with an apex at perfluorooctanoic acid (PFOA). Test results suggest the difference in accumulation of PFAAs in the liver is responsible for the different hepatic responses observed between analogues with different C-atom chain lengths. Moreover, it is generally agreed that PFOA, being the most potent analogue, is the best source compound for filling the data gaps within this category. Specifically, the NOAEL for PFOA is 0.6 mg/kg bw/d based on hepatocyte necrosis (males) and hepatocellular hypertrophy and increased liver weight (females).

Uncertainty of the read-across is reduced with testing within ToxCast which provides data for six of the seven category analogues. Analysis of these data reveals that only ten to thirty of the more than 600 assays performed within ToxCast exhibit activity. The assays with positive results included those related to PPAR and estrogen receptor activity. The potential for PFAAs to undergo PPARg binding was predicted by a COSMOS-developed *in silico* docking profiler. Taken collectively, these new methods supports the premise that the molecular mechanism of action that induces repeated dose liver toxicity of PFAAs is PPAR-linked. Reading across from the most potent analogue, PFOA, to the other analogues within the C_6 to C_{12} category is a conservative prediction.

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3.5.2 *Ab initio* Case Study

Elisabet Berggren

The planning of the *ab initio* case study was commenced in a workshop organised by the JRC in Ispra, 9-10 October 2014. The aim of the workshop was to achieve a common understanding and a better definition of the *ab initio* case study, identify data contributors and formulate a project plan. The workshop participants agreed that hepatotoxicity is the driving toxicity in this case study. A test compound must be defined and the dose range should be identified by an exposure scenario of a daily-applied body lotion. From the applied dose, an internal dose will be predicted, starting with a model estimating how much of the substance would penetrate the skin. The focus is then on the needs of the risk assessor to: (i) determine *a priori* the human-relevant modes-of-action of primary concern; and (ii) define a relevant quantitative point of departure for repeated dose in relation to the exposure scenario indicated. As such, the prediction for the safety assessment is based only on alternative data. This approach has limited relevance regarding safety assessment decisions and is more designed to demonstrate what can be achieved with an approach based solely on data from non-animal methods and, thus, illustrates overall progress made in **SEURAT-1** and highlights gaps for future research.

It was further agreed to start with a simple PBPK using already existing data and then further refine the model if possible. The point of departure for a quantitative assessment would be based on the fact that in each test system at least two doses would be applied, one based on the realistic predicted exposure and one showing the effect based on the expected mode-of-action. This would indicate whether the test system is able to show expected results and if a realistic dose of exposure would be of concern. To estimate the actual *in vitro* concentrations, it was also agreed to analyse lysate concentrations for the test methods contributing to the case study. The workflow describing the steps to complete the *ab initio* case study is summarised in *Figure 3.4*.

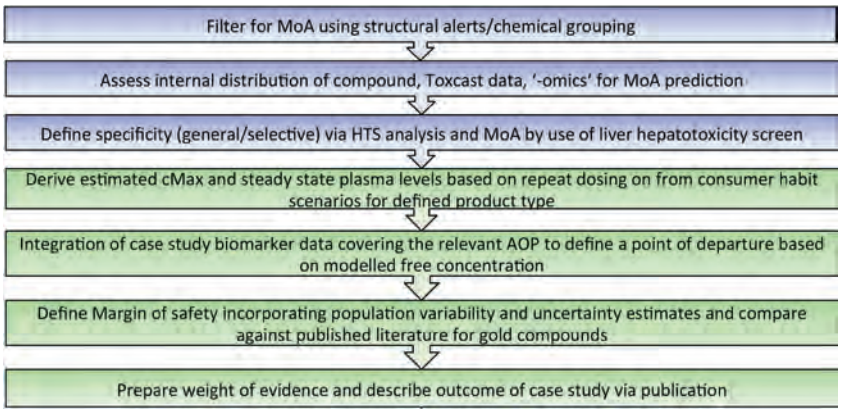

Figure 3.4 *Workflow of the* ***SEURAT-1*** *ab initio case study.*

In the follow-up of the workshop it was agreed to use the two **SEURAT-1** standard reference compounds methotrexate and valproic acid (VPA) as positive controls for fibrosis and steatosis, and one cosmetics relevant ingredient as a test compound. Piperonyl butoxide (*Figure 3.5*) was chosen as the test compound as it had been used in skin cream and shampoo, and it is a known hepatotoxin, even though the mechanism of action is unknown.

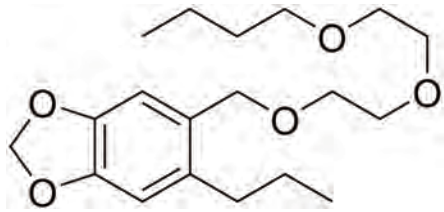

Figure 3.5 *Chemical structure of the selected test compound piperonyl butoxide (5-[2-(2-butoxyethoxy)ethoxymethyl]-6-propyl-1,3-benzodioxole).*

The **SEURAT-1** projects *HeMiBio*, NOTOX and DETECTIVE will provide data to the case study. The three compounds will also be further characterised in the methods developed for structural alerts and profiling in COSMOS. Cosmetics Europe, the Biokinetics Working Group and the JRC will coordinate and assist in finalisation of the case study.

3.5.3 Threshold of Toxicological Concern

Chihae Yang, Mark T.D. Cronin, Elena Fioravanzo, Judith Madden, Andrew Worth, Stéphane Vidry, Andrea Richarz

The Threshold of Toxicological Concern (TTC) approach is a non-testing method that allows a preliminary risk assessment of chemicals based on the availability of reliable exposure

information. It is an exposure threshold for chemicals, below which there is a low probability of an appreciable risk to humans. The concept was originally based on the efforts of the Center for Food Safety and Applied Nutrition (CFSAN), at the US Food and Drug Administration (FDA), to address the challenges in the safety assessment of food contact substances. When there are no data available for a target compound for use at low exposure, TTC can be a pragmatic assessment tool. It is currently used for evaluation of flavouring agents by the FAO/WHO Joint Expert Committee on Food Additives, as well as for the evaluation of genotoxic impurities in pharmaceuticals and natural health products. There is increased interest in broadening the use of the TTC concept within the regulatory context. However, the fact that the original TTC approach is based on data from oral toxicity testing studies is a critical issue for applications with different exposure routes, as in the case of cosmetics. This concerns both the chemical space of the TTC concept developed from oral exposure studies as well as the extrapolation of exposure routes and related bioavailability differences.

The COSMOS project within the **SEURAT-1** Research Initiative has supported the evaluation and extension of the TTC approach to cosmetics ingredients and chemicals in formulations. In collaboration with ILSI Europe, COSMOS has set up two expert groups to deliver opinions and recommendations to guide COSMOS with these efforts. The two expert groups addressed two major tasks. The first Expert Group contributed to the creation of a new non-cancer TTC dataset of No Observable Adverse Effect Levels (NOAELs) with the aim to enrich the existing TTC dataset with cosmetics ingredients and chemicals used in cosmetics formulations. The majority of the NOAEL data in the COSMOS TTC dataset has been compiled from regulatory sources and stored in the COSMOS oral repeated dose toxicity database (oRepeatTox DB). This work included the planning and execution of three different rounds of quality control through numerous study reviews by the external experts. The second Expert Group evaluated oral-to-dermal extrapolation and addressed the difference in exposure scenarios and bioavailability issues, such as dermal absorption and skin metabolism. Research into skin permeability has been supported by collation of skin permeability data, which will ultimately be implemented in COSMOS DB, as well as the redevelopment of models for the calculation of permeability coefficients. A decision-making approach has been devised that comprises various steps to evaluate whether the TTC methodology can be applied to dermal exposure. A number of examples for use case scenarios for cosmetic-relevant materials are being developed to illustrate the applicability of the approach.

Overall there are two goals in the TTC case study: to use the databases for TTC values and skin permeability; and to apply the new methods to confirm that the approach is practical and yet scientifically sound enough to be useful for both regulatory bodies and industries.

4 THE PROJECTS

"The characteristic of scientific progress is our knowing that we did not know."

Gaston Bachelard

4.1 Introduction

Tilman Gocht

This chapter provides a comprehensive overview of the projects of the **SEURAT-1** Research Initiative and, thus, generates the backbone of the Annual Report. Even though the focus is now shifting towards the case studies reported in chapter 3, one should keep in mind that the work programmes of the **SEURAT-1** projects were formulated independent from these case studies. Hence, there is much progress in the different projects outside of the **SEURAT-1** case studies, and this is reported in the subsequent sections with stories about the scientific achievements in the **SEURAT-1** projects, summarised in the respective 'highlight' sections in 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 of the projects started on 1 January 2011. The first volume of the Annual Report focused on the plans and challenges of the different projects and the second and third volumes contained initial results from the research conducted within the **SEURAT-1** Research Initiative. This was continued in the fourth volume and complemented with the contribution of the projects to the **SEURAT-1** proof-of-concept case studies, which were formulated in the meantime. We are keeping this structure for this fifth Annual Report and present the research highlights from the last year in the context of the overall progress within the projects. Furthermore, each project description includes the following sections: (i) the innovative aspects with respect to the achieved results; (ii) the established cooperation with other projects in the **SEURAT-1** Research Initiative; (iii) the expected progress for the last year of the project; and (iv) future long-term perspectives, describing possible next steps based on achieved and expected results from the various projects. An overview of the Principal Investigators from each institution, organised within the projects, completes these sections.

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

These cross-cluster activities are emerging more and more over the lifespan of the **SEURAT-1** Research Initiative. 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 and fourth 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 is briefly summarised and updated in this fifth volume and further complemented with an outline of the '**SEURAT-1** Tools and Methods Catalogue', which, together with the research data stored within ToxBank, is intended to become a sustainable resource for future research programmes operating in the field of predictive toxicology. Overall, this section outlines how **SEURAT-1** as a whole is navigating towards achieving the final goal, which 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. Of course, the Working Groups play a major role in these efforts and reports on activities and workshops conducted under the umbrella of the Working Groups complement this section. In total, the following six Working Groups were 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 are populated with members from different projects, enabling targeted discussions on the needs and contributions of the **SEURAT-1** research projects to meet the cluster-level objectives. Additional workshops were organised based on 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 the outreach activities finishes this chapter. The central aspects here are: a report from the second **SEURAT-1** Summer School (held in June 2014); dissemination activities at conferences; the **SEURAT-1** public website; and the adoption of a dissemination strategy targeting increased coverage of **SEURAT-1** activities in the mass media, which includes the involvement of professional journalists and the establishment an internal Editorial Review Board. Besides the Annual Report, these activities are now the most important cluster-level tools to promote the dissemination of knowledge.

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

Catherine Bell, Gustav Holmgren, Henrik Palmgren, Josefina Edsbagge, Tommy B. Andersson, Magnus Ingelman-Sundberg, Vania Rosas, Marc Peschanski

4.2.1 Introduction and Objectives

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

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

Pluripotent stem cells, whether of embryonic origin (ES cells) (*Thomson et al., 1998*) or

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

➠ 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 5 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) the objectives are:

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

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

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

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

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

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

4.2.2 Main Achievements in the Fourth Year

The 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 format. These achievements allowed us to undertake the second step of the program, and will help to demonstrate the value of pluripotent stem cells derivatives in toxicology studies at an industrial scale.

Several meetings have taken place in order to choose the ‘demonstrators’, i.e. the full

assays that will be developed in order to meet the requirements of the industrial platforms and constraints. These have led to the choice of two major lines of activities, both based on genetically engineered iPS cell lines expressing a reporter for activity of the Nrf2 transcription factor, as a marker of cell responses to oxidants. The first one explores responses of keratinocytes and pluri-stratified epidermis. The second one analyses those of neural cells. Both lines of activities are based upon well-established differentiation protocols that make cells amenable to large-scale screens on industrial platforms. One iPS cell line has been engineered. Different assay systems that meet the requirements of the programs have been set-up.

4.2.3 Selected Highlight: Novel Systems Derived from Stem Cells Aimed at Predicting Hepatotoxicity Induced by Chemical Compounds

Introduction and State of the Art

The liver is the central organ metabolising xenobiotics, and as such is also susceptible to damage by chemicals that enter the body. Many chemicals that cause liver damage in humans are not liver toxic in e.g. rodents, indicating the importance of human relevant test systems to assess safety aspects of chemicals.

Liver toxicity is multi factorial, and in many cases specific to the individual since it only occurs in a small fraction of the population (idiosyncratic toxicity). The complexity of developing reliable and predictive *in vitro* models for liver toxicity is enormous since the biological basis for most of liver toxic compounds is still unknown. Variation in host metabolic functions, detoxification, liver regeneration and immune-response pathways have however been implicated (*Lee, 2003*). *In vitro* systems should therefore be able to reflect a variety of responses including formation of reactive metabolites, mitochondrial functions, interruption of bile flow, formation of immune targets, formation of antibodies (antibody-mediated toxicity), direct cytotoxicity, T-cell responses and secondary immune responses that can cause inflammation. A single *in vitro* model will not cover all possible mechanisms of liver toxicity. Current models focus on toxic effects to vital cell functions mediated directly by the compounds or their metabolites.

Current techniques to assess liver toxicity *in vitro* include cell models such as cell lines (e.g. HepG2) and primary human hepatocytes alone or in co-culture. Primary human hepatocytes (PHH) at present represent the best system for predicting chemically induced hepatotoxicity. They express to a great extent relevant enzymes and transporters but studies have clearly shown that both drug metabolising enzyme and drug transporter functions rapidly decline in 2D cultures and therefore toxicity related to metabolism or interaction with critical drug transporters such as bile acid efflux functions cannot be reflected (*Ulvestad et al., 2011*). In addition, PHH are expensive and of limited availability, meaning that experimental conditions

cannot always be replicated. Hepatocyte-like cells derived from stem cells, therefore, represent an extremely attractive prospect for the pharmaceutical industry, particularly if systems are developed that allow the preservation of the cells in a differentiated state. Cell lines could in theory be generated from individual patients susceptible to a particular drug toxicity or with a specific metabolic profile, and expanded indefinitely. This would allow the investigation of host-specific factors and provide standardised cellular assays that could be applied to large numbers of compounds. Recently developed protocols have yielded significant improvements to the phenotype of hepatocytes derived from hiPS and hES cell lines, however it remains to be seen how well these cells perform in a toxicological setting.

Approach

In order to assess the performance of recently produced stem-cell derived hepatocytes, a comparison to other currently used models is essential. In addition to comparing the expression of drug metabolising enzymes and other hepatic markers, it is also important to characterise how the cells respond to toxic insult and whether relevant signalling pathways exist and are activated. How closely do hiPS cell-derived hepatocytes reflect the *in vitro*, and more importantly *in vivo*, behaviour?

Chronic models of toxicity are also of particular interest, because of the often delayed-onset of hepatic reactions. Liver toxicity usually doesn´t appear until weeks or months of exposure *in vivo*. To mimic the *in vivo* situation the exposure time should, thus, be longer than the 24-48 hours often used in regular 2D cytotoxicity studies. In our studies, long-term, repeated dosing regimens have therefore been designed and implemented.

We have benchmarked the currently available stem cell lines against both primary cells and cell lines. In addition to HepG2 cells, which are commonly used for large-scale industrial screening of compounds, we have also investigated the HepaRG cell line, which may perform better due to an increased expression of drug metabolising enzymes, in particular CYP3A4. HepaRG is a cell line that maintains many liver-like functions over time in culture and would thus be suitable for long term exposure to toxicants (reviewed by *Andersson et al., 2012*). Utilising HepaRG cells in long term cultures ,i.e. >7 days cultures, remains to be investigated.

For chronic experiments with primary human hepatocytes, spheroids were employed in order to provide a more stable hepatocyte phenotype. The majority of experiments with primary hepatocytes include a very short compound exposure, due to the rapid loss of drug metabolising enzyme function upon plating. Culturing hepatocytes in 3D maintains cell-cell contacts, improves morphology and prolongs survival (*Gunness et al., 2013*).

In addition to an initial comparison of the sensitivity of each cell model, a transcriptomic study is also planned in order to identify shared toxicity pathways which are activated following repeated, low-dose exposure to hepatotoxic compounds.

Results

Differentiation of stem cell-derived hepatocytes: Protocols for the differentiation of hepatocyte-like cells were developed by Takara Bio Europe. Resulting cells formed a uniform monolayer when plated in 2D, showed a characteristic polygonal morphology and expressed hepatic markers such as CK18 and HNF4α (*Figure 4.1; Holmgren et al., 2014*). Cells were also able to store glycogen.

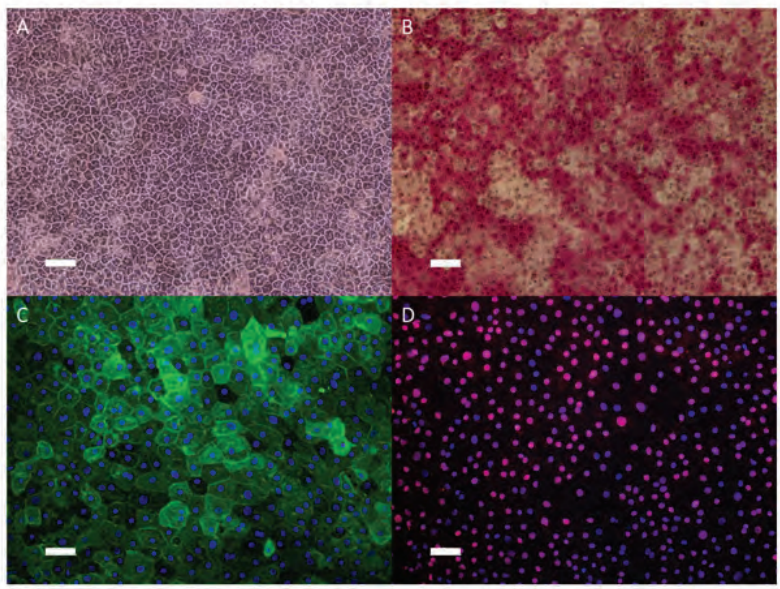

Figure 4.1 *hiPSC-dervied hepatocytes plated in 2D monolayers have a polygonal morphology (A) and store glycogen (B) (Scale bar = 100µm). They also express hepatic markers such as CK-18 (C) and HNF4α (D) (Scale bar = 50µm) (source: Holmgren et al., 2014).*

Metabolic activity of stem cell derived hepatocytes: The expression and function of important CYP enzymes and transporter proteins in hESC-Hep and hiPSC-Hep in comparison to plated cryopreserved PHH was investigated by the application of enzyme-specific substrates and the analyses of the respective metabolites. The results are summarised in *Figure 4.2* and are also reported in *Ulvestad et al. (2013)*. As shown in *Figure 4.2*, CYP activities in hESC- and hiPSC-derived hepatocytes were lower than in PHH cultured for 4 h. On the other hand CYP1A and 3A activities were comparable with levels in PHH cultured for 48 h. Importantly, in hESC-Hep and hiPSC-Hep CYP activities are stable or increasing for at least one week in culture. Analyses of mRNA expression and immunocytochemistry supported the observed CYP activities and showed expression of additional CYPs and transporters in the stem cell-derived hepatocytes (data not shown). In conclusion, the stable expression and function of CYPs in hESC- and hiPSC-derived hepatocytes for at least one week constitutes a basis to perform long-term studies utilising a stem cell-derived hepatic system.

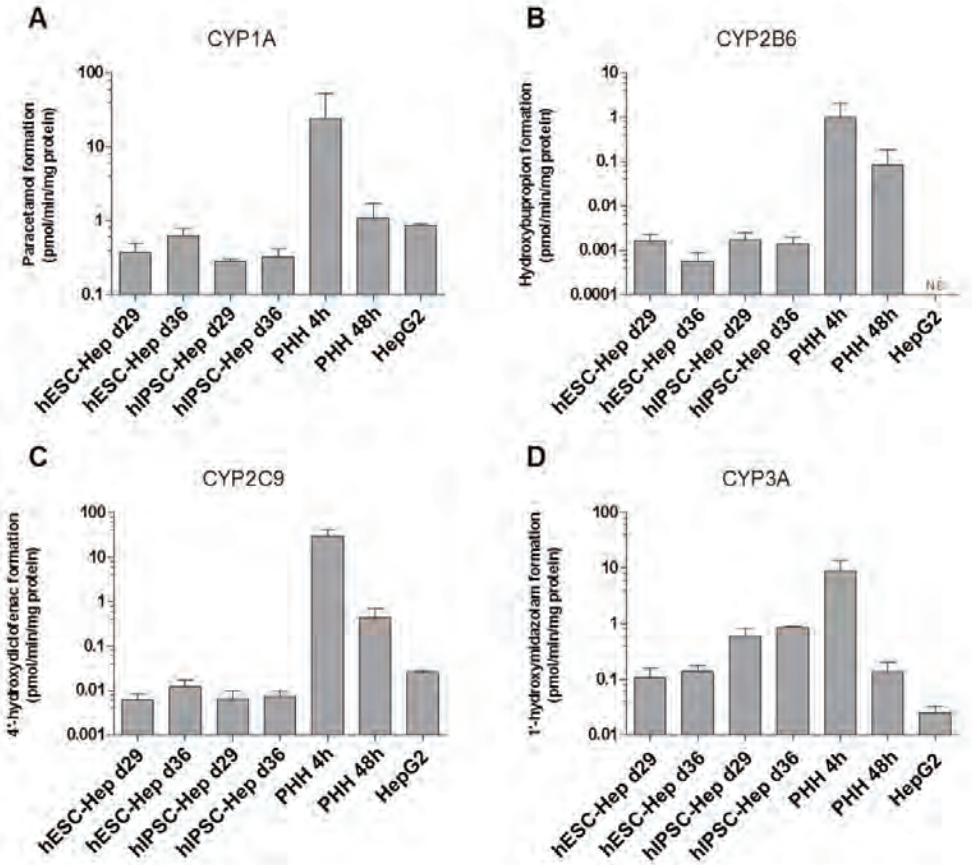

Figure 4.2 *Enzyme activity of CYP1A (A), CYP2B6 (B), CYP2C9 (C) and CYP3A (D) in hESC-derived hepatocytes (hESC-Hep, d29 and d36), hiPSC-derived hepatocytes (hiPSC-Hep, d29 and d36), plated cryopreserved primary human hepatocytes cultured for 4 h and 48 h, and HepG2 cells assessed by the formation rate of paracetamol (metabolite of phenacetin), hydroxyl-bupropion (metabolite of bupropion), 4'-hydroxy-diclofenac (metabolite of diclofenac) and 1'-hydroxy-midazolam (metabolite of midazolam), respectively. Standard deviations are given as error bars, n = 3. N.D., not determined. Abbreviations: d29, day 29 in differentiation protocol; d36, day 36 in differentiation protocol (source: Ulvestad et al., 2013).*

Investigation of repeated-dose toxicity in the hiPSC-derived hepatocytes: The exposure protocols to assess repeated dose toxicity were defined according to previous studies reported in the literature (*Olson et al., 2000; Roberts et al., 2014*). The hiPS cell-derived hepatocytes were exposed to relevant concentrations of hepatotoxic compounds for 2, 7, and 14 days using a repeated dosing regime. As shown in *Figure 4.3* this cell model is stable enough to enable at least a 2-week study of drug exposure. This provides a basis for studies using stem cells derived hepatocytes for monitoring responses on a mechanistic level.

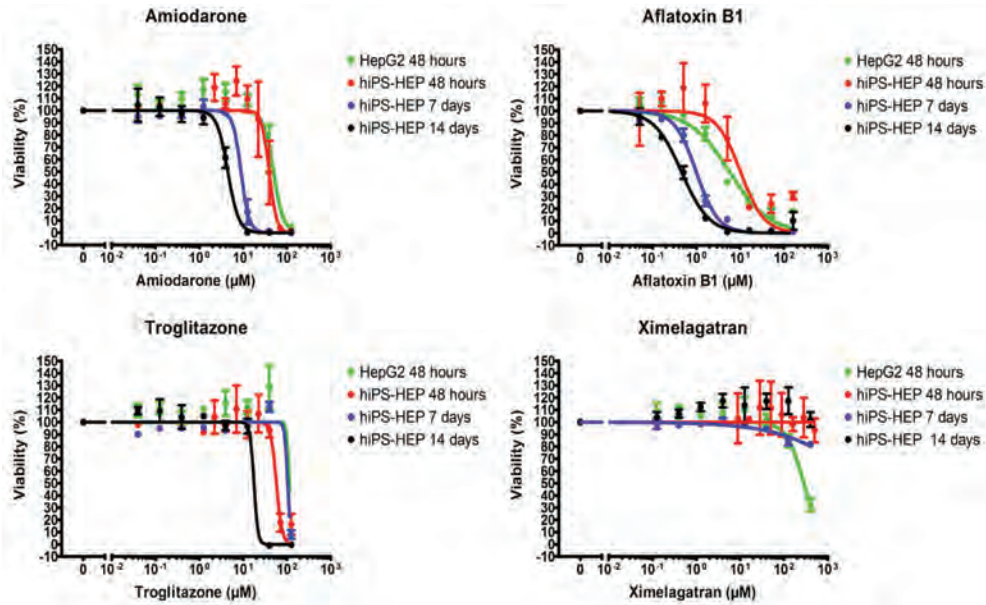

Figure 4.3 *Viability curves of hiPS-derived hepatocytes exposed to compounds for 48 hours (red), 7 days (blue), and 14 days (black), as well as HepG2 exposed to compounds for 48 hours (green). Viability displayed as mean S.E.M. (n = 3) (Holmgren et al. 2014).*

The hiPS-derived hepatocytes became more sensitive to the toxic compounds after extended exposures and, in addition to conventional cytotoxicity, evidence of phospholipidosis and steatosis was also observed in the cells.

Comparison of hiPS-derived hepatocytes with HepaRG and PHH spheroids: Primary human hepatocytes cultured in 2D lose rapidly drug metabolising enzyme and transporter functions. Therefore, in order to make the most relevant comparison to the long-term exposures performed with hiPS-derived hepatocytes, a more stable method of culturing PHH was developed. Culturing cells in 3D allows the maintenance of cell-cell contacts and results in an improved phenotype. In order to allow multiple conditions to be tested simultaneously and to allow a more high-throughput approach, the spheroid model was selected. Spheroids were generated by culturing primary hepatocytes in 96 well ultra-low attachment plates. The expression of a number of hepatic markers and CYP drug metabolising enzymes was investigated via immunohistochemistry. In most cases, protein expression was very stable for up to 3 weeks post-seeding (*Figure 4.4*), indicating that the spheroids would remain functional throughout the duration of the repeated dose experiment.

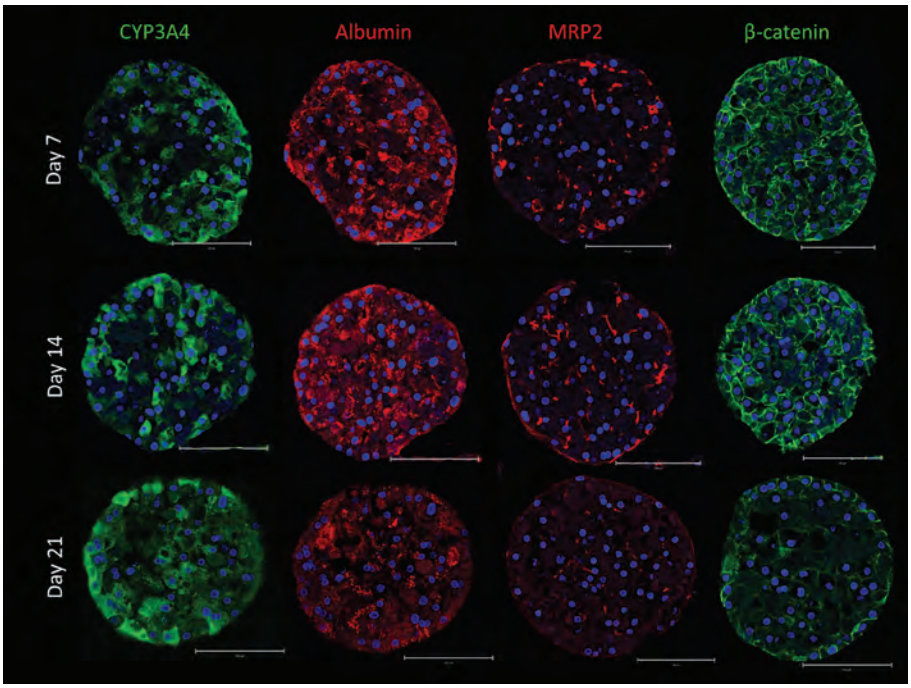

Figure 4.4 *Expression of a panel of hepatic markers is maintained during 3 week culture of primary human hepatocytes in 3D spheroids. Primary human hepatocytes (1500/well) were seeded in ultra-low attachment plates and collected and fixed at the indicated time-points.*

A comparative study was designed utilising a second hiPS-Hep donor (cultured at Takara Bio Europe), the HepaRG cell line (cultured at AstraZeneca) and PHH spheroids (cultured at Karolinka Institutet). Six hepatotoxic model compounds were selected from the **SEURAT-1** standard reference compound list (see section 4.10.4). These were amiodarone, acetaminophen, aflatoxin, chlorpromazine, troglitazone and ximelagatran. Each of the cell systems was exposed to a range of concentrations of each compound every second day. At 48h, 7 days and 14 days a viability assay was performed and the EC_{50} calculated. The EC_{50} values obtained from this study for all cell types are summarised in Table 1. In the majority of cases a reduction in EC_{50} was observed following repeated dosing. The relative sensitivity of each cell type varied for each compound, though in most cases PHH were the most sensitive. The hiPS-derived hepatocytes performed well although they were quite resistant to APAP toxicity and short term treatment with aflatoxin resulted in a 9-fold and 18-fold increase in EC_{50} compared to HepaRG and PHH spheroids, respectively (*Figure 4.5*). An EC_{50} could not be calculated at any of the time-points for ximelagatran, however this might be resolved by increasing the maximum dose to 1000 µM. For troglitazone, chlorpromazine and amiodarone the hiPS-Hep were more sensitive than HepaRG

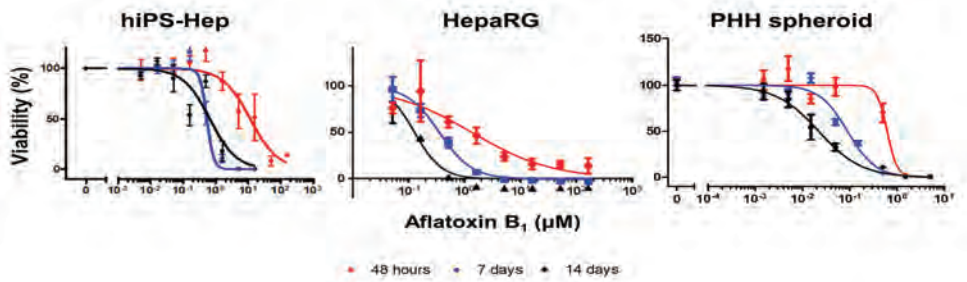

Figure 4.5 *Viability curves for hiPS-Hep, HepaRG and PHH spheroids exposed to Aflatoxin for 48h (red), 7 (blue) and 14 days (black) (n=3).*

Table 4.1 *Summary of EC_{50} values obtained from repeated dose studies performed at Takara Bio Europe (hiPS-derived hepatocytes), Astrazeneca (HepaRG) and Karolinska Institutet (PHH).*

EC_{50} (µM)	hiPS-Hep			HepaRG			PHH spheroid		
	48h	**7d**	**14d**	**48h**	**7d**	**14d**	**48h**	**7d**	**14d**
Aflatoxin B1	11.5	0.5	0.7	1.23	0.35	0.18	0.62	0.09	0.02
Amiodarone	47.6	15.1	15.0	57.9	36.2	23.6	>100	12.3	12.8
APAP	>16000	13964	9439	6025	1944	1717	>10000	1784	131.2
Chlorpromazine	38.4	23.7	24.0	67.0	31.9	35.0	15.5	8.1	5.2
Troglitazone	46.0	33.9	18.7	n/a	143.6	141.5	32.4	4.5	1.4
Ximelagatran	>400	>400	>400	n/a	851	984.7	>1000	476.1	53.38

4.2.4 Innovation

hiPS-derived hepatocytes have not been used previously for repeated dose toxicity studies and the publication by *Holmgren et al. (2014)* represents an important step in assessing the potential of stem-cell derived hepatocytes. As an extension of this work we felt that it was extremely important to provide a comparison to other, existing cell systems, as this is key to understanding how useful the cells could be for use in an industrial setting. The spheroid model is also relatively new, and its benefits are still to be fully assessed.

Although there might be some bias between the systems inherent in differences based on the specific donors of the cells, we can provide some preliminary conclusions from our comparative study. In general for the prediction of toxicity it appears that the HepaRG cell is not markedly more sensitive than the hiPSC-derived system, even though HepaRG cells have a pronounced competence for drug metabolism. This may be an inherent feature of

the tumour phenotype, which causes a lack of expression of relevant signal transduction systems of importance for the mechanisms of chemical induced toxicity. The PHH spheroids showed the highest sensitivity, in particular for the longer periods of treatment (14 days). The 3D configuration of the test system may thus in itself be of importance for the response to a toxicological pathway. Indeed, HepaRG cultivated in spheroids are considerably more sensitive to APAP toxicity than corresponding 2D cultures (*Gunness et al., 2013*).

Concerning the hiPSC-derived hepatocyte system, it is evident that the cells may be used under stable conditions for 14 days and that relevant toxicity outputs can be achieved during this period of time (*Table 4.1*). The profiles were not too far from those generated in the 3D based PHH spheroid system. Indeed, continuous efforts are made to improve the phenotype of such stem cell-derived hepatocytes and it might be a possibility that the cells can be used already for certain applications within an industrial context. Of course a lot of characterisation work is still required in order to define their specific phenotypes, including the mechanisms of chemical induced toxicity, but the result of our comparative study performed within ***SCR&Tox*** encourages further developments of such cell systems and suggests that they already at this point might contribute to toxicity screening programs within industry. The possibility to produce such cell lines from individual patients susceptible to a particular drug toxicity or with a specific metabolic profile, and expand them indefinitely adds to the future utility of such cell systems. In the long run, it might also be possible to introduce non-parenchymal cells derived from the same donors, thereby forming a more complete design of liver organoids derived from stem cells suitable for chronic toxicity studies, in which the contribution of blood cells, immune cells and the interplay with inflammatory cells can be investigated.

4.2.5 Cross-Cluster Cooperation

Several inter-networks meetings have been organised over the past year in order to exchange data and expertise on cardiomyocyte and hepatocyte differentiation, that had been obtained since the beginning of the ***SCR&Tox*** project.

4.2.6 Expected Progress within the Fifth Year

The assay for oxidative stress is being developed in two additional directions, namely for testing keratinocytes in 2D and 3D, and neural cells. First, an iPS cell line has been engineered using a construct provided by the biotech company Givaudan, which has used it already for deriving another type of test called “keratinosens”. Technology transfer is now being organised toward the platforms of the industry partners of the network, in order to move the assay toward the work-package responsible for translation toward industrial platform (see below). Second, all documentations needed for submission of the assay to the regulatory authorities will be prepared. We expect – providing the assay performs as well as planned – to be able to submit an application at the end of the ***SCR&Tox*** project.

4.2.7 Future Perspectives

The expected success of the two final workpackages of the ***SCR&Tox*** network will allow us to demonstrate the amenability of cells derived from pluripotent stem cell lines for testing oxidative stress response to a toxicant. These demonstrations will bring a proof-of-concept at two levels, biological and industrial. Biologically, the promise of pluripotent stem cells will be confirmed through two different cell types that can be produced and explored at will at a scale that matches the need of industry. Industrially, the amenability of the cells to miniaturisation and automation will open the path for replicating the technology transfer performed within the ***SCR&Tox*** network in a wider spectrum of industry platforms.

How these tools can open new paths for predictive toxicology altogether, besides facilitating the pre-clinical testing of drugs in development is still hypothetical at this stage. As suggested in the previous **SEURAT-1** Annual Report (*Rosas et al., 2014*), one interesting development is that the use of iPS allows to seek not only pre-clinical data but also, through specific clinical-biological iteration, direct biological correlates of clinically observed drug toxicity. The proposed paradigm would consider *in vitro* toxicology as a complement to clinical evaluation by comparing molecular mechanisms triggered by a drug in relevant cell phenotypes between iPS-derived cells obtained from patients who showed a toxic response and patients who did not. Such an analysis would help focus on adverse outcome pathways and identify susceptibility factors, eventually leading to rescuing drugs that may have otherwise been killed by providing biological markers to sort out patients who should be excluded while maintaining those who may not, and promote the search for companion testing.

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 phase.

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4.3 HeMiBio: Hepatic Microfluidic Bioreactor

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4.3.1 Introduction and Objectives

Refinement, reduction and replacement of animal usage in toxicity tests (the 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 human and animal models.

In ***HeMiBio***, we propose to generate a liver-simulating device mimicking the complex structure and function of the human liver. The device will reproduce the interactions between hepatocytes and non-parenchymal liver cells (hepatic stellate, sinusoidal endothelial, and Kupffer cells) for over one month *in vitro*. Such a Hepatic Microfluidic Bioreactor could serve to test the effects of repeated exposure to chemicals, including cosmetic ingredients. To create such a device the cellular components of the liver need to be viable for over one month, with *in vivo*-like metabolic and transport 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 4.6*) 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.

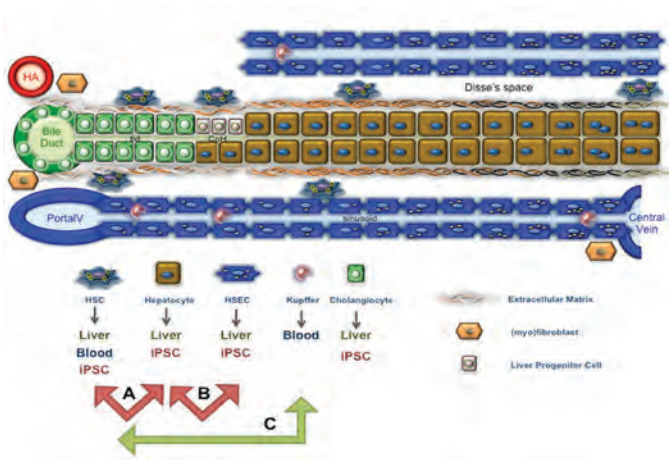

Figure 4.6 *Schematic representation of a liver sinusoid (adapted from: Dollé et al., 2010).*

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.

4.3.2 Main Achievements and Challenges in the Fourth Year

During the last year, extensive focus was on: (i) further optimisation of the culture methods and subsequent characterisation of hepatic stellate cells and liver sinusoidal endothelial cells; (ii) the differentiation of pluripotent stem cells towards hepatocytes; (iii) testing of the **SEURAT-1** standard reference compounds on hepatocytes treated with the UpCyte® technology; (iv) further 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 **SEURAT-1** case studies (see sections 3.4.1 and 3.5.2).

Optimisation of the Culture Methods and Subsequent Characterisation of Hepatic Stellate Cells and Liver Sinusoidal Endothelial Cells

As described below (see section 4.3.3), we have completed the molecular and epigenetic profiling of primary liver derived hepatic stellate cells. These data were presented at the fifth

SEURAT-1 Annual Meeting in January 2015 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. As described in the case report below (see section 4.3.4), we are also able to generate co-cultures between the cell line HepaRG and primary human 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

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 4.10.4).

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 laboratories 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. The design of this approach is shown

in *Figure 4.13*. A manuscript is under revision 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 4.3.3), 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.

4.3.3 Selected Highlights: Integration of Electronic Sensors, Real-Time Oxygen Measurements, Toxicity Testing, Genome Engineering and Genome-Wide Profiling of Promoter DNA Methylation

Integrated Electronic Sensors for Cell Culture Status Monitoring

A set of sensors for the non-invasive monitoring of the metabolic status of the *in vitro* liver tissue model has been developed and tested with the ***Hemibio*** bioreactor. Based on observation of the biological tissue model and considerations regarding, the following extracellular chemical biomarkers, secreted or excreted by the cells were selected:

- pH, pO_2, glucose, lactate for active control and regulation of the cell culture conditions and response of cell culture to toxic insult;
- K^+, NH_4^+/urea, ALT for response of cell culture to toxic insult.

The absence of cross-talk between the sensors mounted in series in the same fluidic setup has been proven using glucose, lactate and glutamate sensors. These same sensors were used to follow the growth of a healthy HepG2 cell culture over several hours. From *Figure 4.7* the consumption of glucose, followed by the augmentation of both lactate and glutamate are visible.

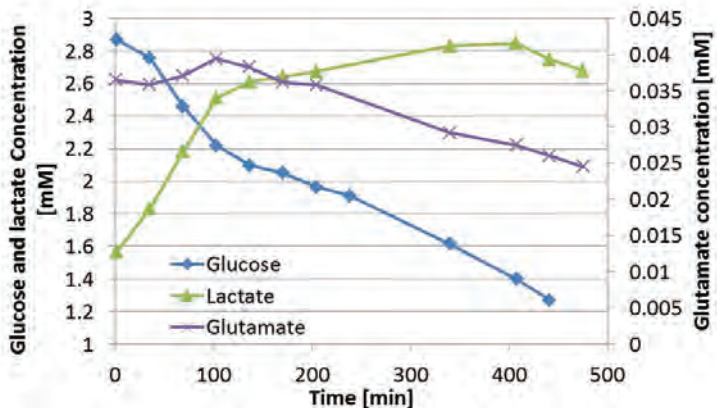

Figure 4.7 *Concentration profile of glucose, lactate and glutamate in a HepG2 cell culture.*

Following the proof of the feasibility of the monitoring of the cell culture status of an *in vitro* model, the sensor module, including glucose, lactate, ALT electrochemical sensors and fluorescent bead pO_2 sensors has been connected to the ***HeMiBio*** bioreactor. The schematic of the automated test setup is shown in *Figure 4.8*.

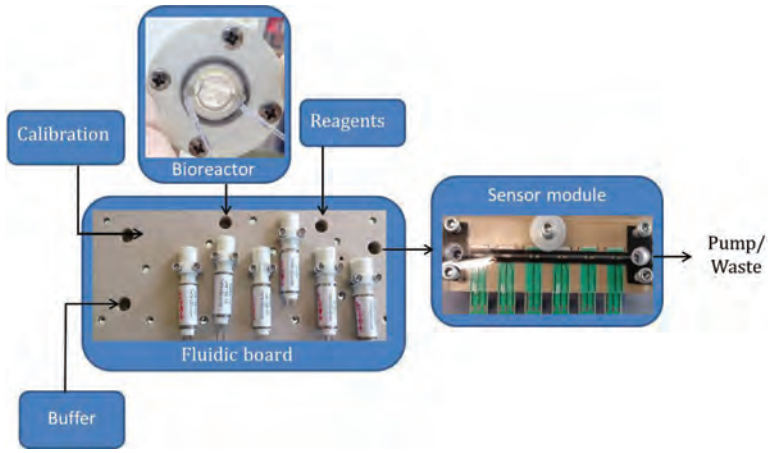

Figure 4.8 *Schematic of the automated bioreactor monitoring setup.*

HepG2 cells were cultivated and monitored in the 40 ml bioreactor for 21 hours before exposing the cells to the toxic compound rotenone at a concentration of 200 mM. Concentrations profiles of glucose, lactate, pO_2, ALT were successfully recorded for five hours after the toxic insult with a sampling rate of 1 sample/hour. The cell necrosis was clearly visible by the lowering of oxygen consumption by the cells. At the same time, the ALT concentration signal showed a raise (*Figure 4.9*). This preliminary result on ALT will have to be confirmed with further testing.

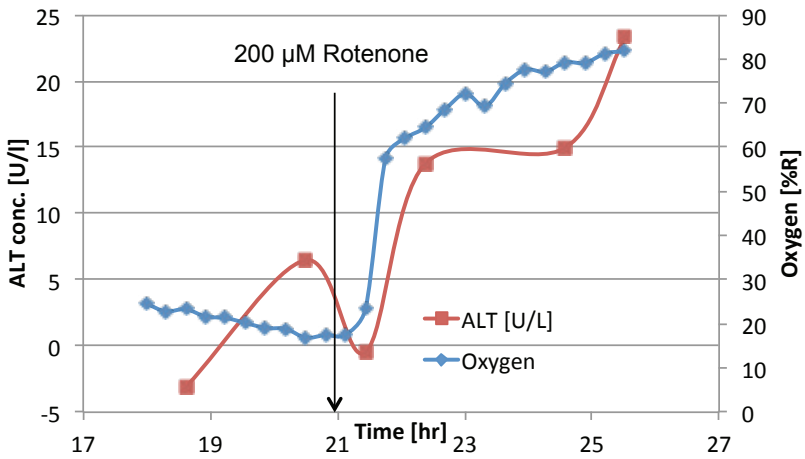

Figure 4.9 *Concentration profile of pO_2 and ALT in the* ***Hemibio*** *bioreactor with a HepG2 cell culture before and after the exposition of the cells to 200 µM rotenone.*

Real-Time Focus Independent Measurement of Oxygen Uptake in Microfluidic Bioreactor

Oxygen uptake is a critical measurement of mitochondrial function and metabolic activity (*Green & Reed, 1998; Han et al., 2013*). However, electrochemical electrodes require frequent re-calibration, while optical sensors are affected by small changes in focus, cell migration and stage movement, making them unreliable for *real time* measurements of oxygen uptake. Therefore, we designed an optical system in which organoids are embedded with nanoprobes containing RuP dye and excited at 532 nm (*Figure 4.10*). Oxygen is a quencher of RuP phosphorescence, leading to a decrease in decay time. Importantly, decay time is not sensitive to changes in probe concentration or excitation intensity (i.e. cell migration, mechanical movement; *Figure 4.10*). To measure in *real-time,* we used a novel two-frequency sinusoidal intensity-modulated light, allowing us to filter background interference while measuring phase shift in the emitted light. Measurements were taken every 15 minutes for 28 days with no photo-toxicity, signal drift, or relevant loss of intensity.

Importantly, our *real-time* high sensitivity measurement of oxygen uptake uncovered a novel mechanism of acetaminophen toxicity. We showed that acetaminophen causes an immediate, reversible, dose-dependent loss of oxygen uptake followed by a slow, irreversible, dose-independent death (*Figure 4.10*). Importantly, the high sensitivity of our system allowed us to detect a minute transient loss of mitochondrial respiration below the threshold of acetaminophen toxicity. The phenomenon was repeated in HeLa cells that lack CYP2E1 and 3A4, and localised to mitochondrial C3, suggesting a NAPQI-independent mechanism might

be responsible for dermal and renal toxicity *(Prill et al., 2015)*. These results indicate the importance of tracking toxicity over time.

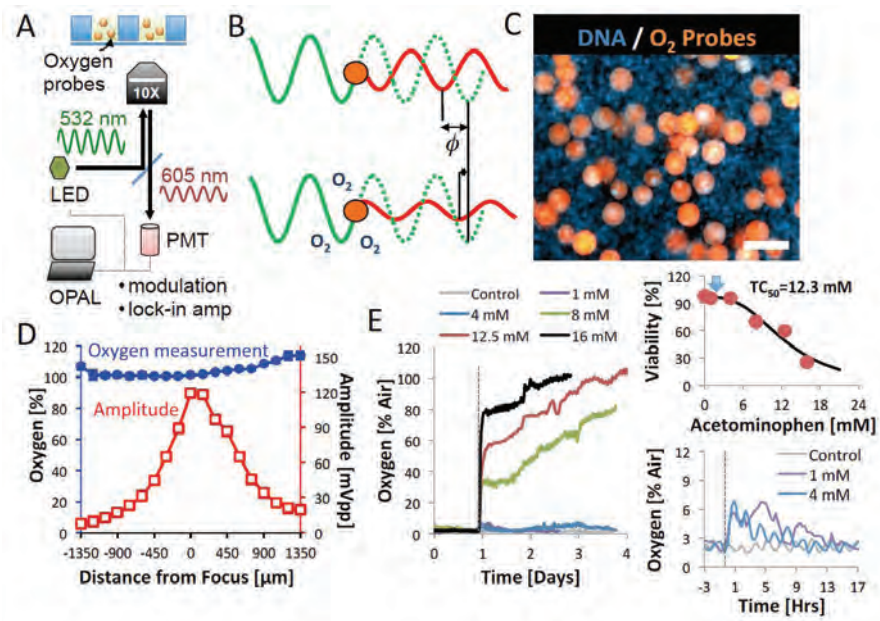

Figure 4.10 *(A) System schematic. (B) Oxygen-quenched decay causes phase shift between the intensity-modulated excitation and emission light. Two-superimposed frequencies were used to screen out background interference. (C) Fluorescence image of HepG2/C3A cells and sensor particles after immobilisation in a collagen matrix. (D) Fluorescence intensity is rapidly lost away from the plane of focus, while phase shift measurements are unaffected. (E) Oxygen uptake over time response of HepG2/C3A cells exposed to acetaminophen. An immediate dose-dependent loss of oxygen uptake concludes in 60 min, while a slow dose-independent loss of oxygen uptake terminates with total cell death within 3 to 5 days. Top right: Dose dependence of acetaminophen after 12 hours.* TC_{50} *was calculated to be 12.3 mM. No effect is observed at 4 mM acetaminophen at 12 hours (blue arrow). Bottom right: Temporal close up shows a transient loss of mitochondrial respiration at 1 and 4 mM acetaminophen, below threshold at which cell death could be detected (source: Prill et al., 2015).*

Single Dose Toxicity Gold Compound List

Dose response toxicity was assessed using LiveDead staining as read-out of acetaminophen, acetylsalicylic acid, amiodarone, chlorpromazine, cyclosporine A and diclofenac toxicity were performed are summarised in *Table 4.2*. Susceptibility for d20 hepatocyte like cell progeny from PSC was comparable to published data in literature.

Table 4.2 *PSC-derived hepatocyte like cells: acute toxicity profile.*

	TC_{50}	R^2	Literature
Amiodarone	**805 μM**	0.9998	50-100 μM
Acetaminophen	**24 mM**	0.9991	35 mM
Acetylsalicyl acid	**7 mM**	0.9914	20 mM
Chlorpromazine	**102 μM**	0.9935	3 -35 μM
Diclofenac	**1523 μM**	0.9924	400 μM
Cyclosporine A	**35 μM**	0.9987	44 μM
Valproic Acid	**39mM**		16-62mM

We also demonstrated that cyclosporine A causes cholestasis (cytoplasmic retention of CDF-DA) while acetaminophen causes apoptosis (immunofluorescence Caspase-3 staining; *Figure 4.11*).

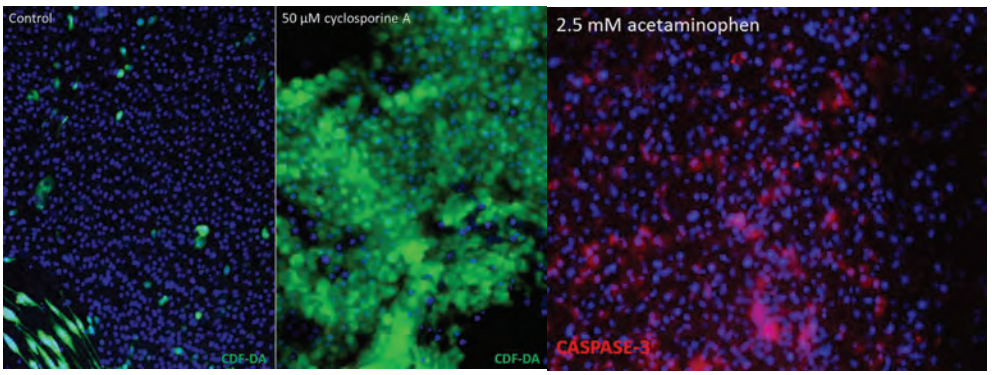

Figure 4.11 *Human PSC exposed to CSA and acetominophen: staining with CDF-DA for cholestasis (left and middle panel) and caspase-3 antibodies for apoptosis (right panel).*

Steatosis was observed after incubation with amiodarone or acetylsalicylic acid. Pathologic lipid accumulation occurred after incubation with 2 μM amiodarone (*Figure 4.12*, panel B) or 500 μM acetylsalicylic acid (*Figure 4.12*, panel C).

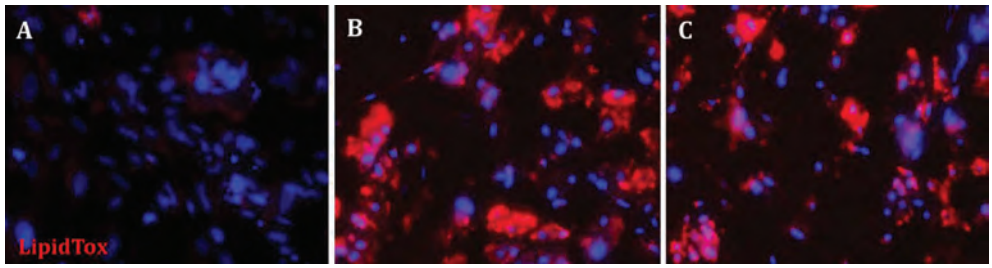

Figure 4.12 *Steatosis following incubation of PSC hepatocytes with amiodarone (B) or acetylsalicylic acid (C).*

Genome Engineering in PSC to Allow Very Fast Incorporation of MoA Cassettes

During the past year, we have generated donor plasmids for lineage tracing, inducible overexpression of transcription factors and inclusion of toxicology readout cassettes which were exchanged with flipase in the 'safe harbour' AAVS1 location previously engineered in pluripotent stem cells by homologous recombination. The design of this approach is as shown in *Figure 4.13*. This approach allows the generation of newly targeted PSC lines within 2 weeks, without the need for single cell isolation.

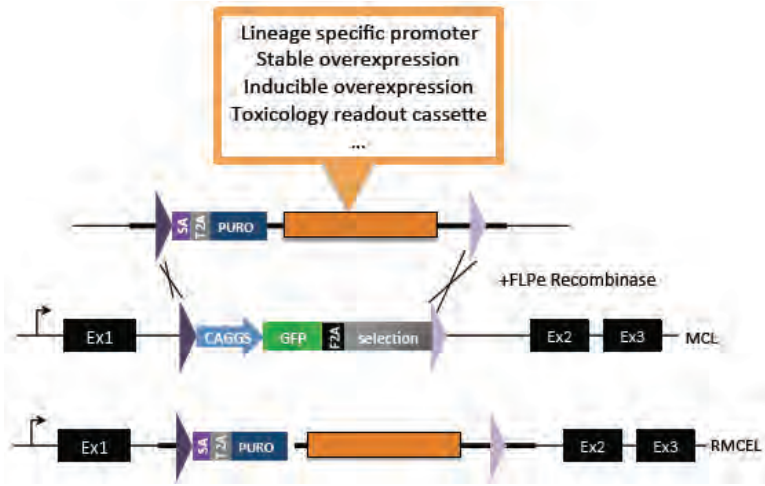

Figure 4.13 *Generation of a master RMCE line, followed by flipase mediated introduction of lineage specific promoters, overexpression and toxicity cassettes).*

A manuscript is under revision describing the possible applications of this technology.

Genome-Wide Profiling of Promoter DNA Methylation in Three Primary, Non-Cultured Liver Cell Types

The human liver contains multiple cell types whose epigenetic patterns are undetermined. We examined the promoter methylome of purified and uncultured hepatic stellate cells (HSCs), hepatocytes (Heps) and liver sinusoidal endothelial cells (LSECs) from several donors. We carried out methylated DNA immunoprecipitation (MeDIP) and promoter array hybridization using a Roche-Nimblegen array spanning 4 kb of all human RefSeq promoters. Uncultured HSCs, LSECs and Heps display ~7000-8000 methylated promoters, with 60-70% similarity between all cell types. Gene ontology for the commonly methylated genes reveals involvement in germ cell development and reproduction, segregating germ-line from somatic lineage methylation. HSCs, LSECs and Heps also contain ~500-1000 uniquely methylated promoters; these are implicated in signalling and biosynthetic processes (HSCs), lipid transport and metabolism (LSECs), and chromatin assembly (Heps). The promoter methylome of culture-activated HSCs deviates from that of uncultured quiescent HSCs. HSC culture also enhances methylation differences between individual donors but this does not always relate to changes in gene expression. Our data provide the first genome-wide maps of promoter DNA methylation in human purified and uncultured liver cell types. Although methylation profiles are largely similar between HSCs, LSECs and hepatocytes, detection of cell type-specific methylation patterns suggests a differential epigenetic programming of these cell types in the liver.

4.3.4 Contributions to the SEURAT-1 Case Studies

An *in vitro* culture where liver fibrosis can be recapitulated in response to a test compound is difficult to obtain, especially in regular hepatic mono-cultures. The combination of hepatocytes and hepatic stellate cells (HSCs) adds the drug biotransformation cells to the main responders of the fibrotic injury (and scar tissue) constituting a more representative approach. Nevertheless, in order to have a good predictive capacity it is mandatory to ensure the viability and functionality of the cells for the entire culture time. The focus of the ***HeMiBio*** case study on the development of a human *in vitro* model that can mimic drug-induced liver fibrosis is discussed in section 3.4.1. During the previous years of the **SEURAT-1** Research Initiative the model was optimised in order to have non-active HSCs and functional hepatocytes (HepaRG) for 21 days. This was achieved by keeping the co-cultured cells in 3D spheroids, which avoids the spontaneous HSC activation normally induced by contact with rigid surfaces (*Olsen et al., 2011*) and at the same time maintains good metabolic functions of the HepaRG (*Leite et al., 2012; Gunness et al., 2013*).

The case study is still on-going and generated data are also relevant for the **SEURAT-1** level 3 *ab initio* and read-across case studies (see sections 3.5.1 and 3.5.2).

4.3.5 Cross-Cluster Cooperation

During the last year close collaborations have been set up with the following groups: (i) between Catherine Verfaillie, *KULeuven*, Leo van Grunsven, *VUB,* and Bob van de Water, *Universiteit Leiden* (DETECTIVE), to perfrom genome engineering of PSC suitable for toxicity testing, specifically fibrosis; (ii) between Leo van Grunsven, *VUB*, and Christophe Chesne, *Biopredic* (NOTOX) to develop organoid cultures suitable for fibrosis assessment using HepaRG cells; (iii) between Leo van Grunsven, *VUB*, and Fozia Noor, *Saarland University* (NOTOX) to adapt the organoid culture into serum-free medium and to perform metabolome studies; (iv) between Catherine Verfaillie, *KULeuven*, and Christophe Chesne, *Biopredic* (NOTOX) to genome engineer HepaRG cells.

In addition, the group of Toni Cathomen, *Universitätsklinikum Freiburg*, was invited to give a presentation on the optimisation of the oxidative stress assay using the NRF_2-tomato reporter developed within ***HeMiBio*** during a *SCR&Tox* meeting in June 2014.

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. In 2014, a list of cosmetic compounds for toxicity testing purposes has been selected by the *Vrije Universiteit Brussel* group and has been shared with the DETECTIVE consortium. 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*** information regarding the selection of compounds. Furthermore, Mathieu Vinken from the *Vrije Universiteit Brussel* group is member of the **SEURAT-1** Safety Assessment Working Group and co-leader of the **SEURAT-1** Mode-of-Action Working Group, both of which working towards the cluster-level objectives of the **SEURAT-1** Research Initiative (see sections 4.10.6 and 4.10.9). Furthermore, as Vice-President of the European Society of Toxicology *In Vitro* (ESTIV) Mathieu Vinken co-organised the **SEURAT-1**/ ESTIV joint summer school in Egmond aan Zee, The Netherlands, on 8-10 June 2014 (see section 4.11.1). Mathieu Vinken was also invited speaker on the NOTOX meeting organised on 10 June 2014 in Egmond aan Zee, The Netherlands. Moreover, in order to make available data obtained within ***HeMiBio*** consortia to the **SEURAT-1** partners, raw data and analysed results have been uploaded into the ToxBank Data Warehouse. To date, partners from *IDIBAPS*, *Vrije Universiteit Brussel*, *Medicyte GmBH* and *Universitetet I Oslo* have uploaded data and standard operation protocols to the ToxBank Data Warehouse.

Furthermore, the following **SEURAT-1** workshops have been attended on behalf of ***HeMiBio***:

- 29-90 April 2014: **SEURAT-1** workshop *"The read-across case study for safety assessment contributing to the SEURAT-1 Proof-of-Concept" in Ispra, Italy;*
- 8-10 June 2014: **SEURAT-1** & ESTIV joint summer school in Egmond aan Zee, The Netherlands;
- 10 June 2014: NOTOX Satellite Meeting, Egmond aan Zee, The Netherlands;
- 9. October 2014: **SEURAT-1** *ab initio* case study workshop.

4.3.6 Expected Progress within the Fifth Year

In year five 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;
- Perform toxicity testing of selected (cosmetic) compounds in the bioreactor;
- Contribute to the **SEURAT-1** ab initio case studies using different cell culture systems available in ***HeMiBio***;
- Participate in the Read Across case study using the HepaRG/HSC co-cultures.

HeMiBio partner *Vrije Universiteit Brussel* has selected a number of cosmetic compounds to be tested in the ***HeMiBio*** reactor, including furfural, benzophenone, solvent red 23, cupric acetate and copper sulphate. These cosmetic compounds are suspected to cause liver fibrosis. They have been selected together with the ToxBank consortium and have been backed up by data found in the SCCS databank previously established by the *Vrije Universiteit Brussel*. However, on the last **SEURAT-1** Annual Meeting in January 2015 in Barcelona it was suggested to test a specific set of compounds in all projects in the final year in the context of the *ab initio* case study (see section 4.9.2) and therefore priority will be given to testing of these compounds, including piperonylbutoxide, valproic acid and methotrexate.

4.3.7 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) bio-artificial 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 bio-artificial 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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El Taghdouini, A., et al. (2015): Development of culture conditions to revert the activated phenotype of cultured human hepatic stellate cells. Poster award at the SEURAT-1 5th Annual Meeting 2015, 21–22 January 2015, Barcelona.

The prizes consists of 500€ voucher contributing to travel expenses to attend a Conference related to **SEURAT-1** research fields.

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

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4.4.1 Introduction and Objectives

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. 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 overall objectives 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.

The specific objectives of **DETECTIVE** during the fourth year were:

- To complement genomic, proteomic and metabonomic data derived by '-omics' studies with cellular responses in order to develop a panel of cytomic assays;
- To identify new potential biomarkers relevant for hepatotoxicity, cardiotoxicity and nephrotoxicity or for other cell types of toxicological relevance;
- To perform quality assessment of biomarkers. To compare sensitivities of applied technologies as well as to assess the specificity and predictivity of identified biomarkers;
- To perform cell type specific readouts, such as albumin or urea secretion, phase I and phase II metabolising activities of hepatocytes, or protein release from cardiomyocytes;
- To test which of the conventional functional readouts are essential and which can be replaced by faster techniques without losing predictive power.

4.4.2 Main Achievements and Challenges in the Fourth 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 studies were focused on developing a complete toxicological profile with the carefully selected compounds based on both functional and '-omics' analyses. 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 based on *in vivo* human data when available and *in vitro* cell functional profiling.

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 as a novel *in vitro* model for hepatotoxicity testing.

The **DETECTIVE** public website (www.detect-iv-e.eu) is regularly updated and the consortium achievements have been presented at scientific conferences such as the WC9 event in Prague on 24-28 August 2014. **DETECTIVE** has contributed to two workshops organised by COACH in support of the **SEURAT-1** Level 3 case studies: the 'Read Across Case Study' in Ispra on 29-30 April 2014 and the 'Ab Initio Case Study' in Ispra on 9-10 October 2014. The **DETECTIVE** partners have also submitted and published a range of scientific (peer reviewed) papers.

Functional Readouts

Four major types of functional biomarker readouts have been evaluated and implemented: electrical activity, impedance measurements, high-content imaging and cell specific measurements. Impedance measurements, electrical activity and high-content imaging were used for cardiotoxicity analysis. Renal toxicity was assessed by application of impedance measurements and other cell specific measurements. In hepatotoxicity assessment a focus was on a high-content imaging of adverse pathways using cell sensors established on a basis of BAC-GFP reporter systems (>50 candidate cell lines). The reporter cell lines have been verified also for 3D spheroid cell systems (could be cultivated up to four weeks) better representing tissue physiology *in vivo*. Additionally, an *in vitro* model for basal bile canalicular dynamics has been established using a sandwich culture. It allows assessing functionality of hepatocytes in sandwich culture by monitoring bile salt transport.

'-omics' Readouts

The overall objective targeted by '-omics' Readouts is to improve traditional biomarkers of toxicity by gathering and integrating data on transcriptomic, proteomic, metabonomic and epigenomic responses to exposure in human *in vitro* models, which will culminate in a novel set of mechanism-based intermediate biomarkers of repeated dose toxicity applicable to evaluate the safety of different substances. **DETECTIVE** is developing intermediate biomarkers for

repeated dose toxicity to the liver, the kidney and to the heart.

All generated data on transcriptomics; DNA methylation, histone acetylation, miRNA expression, and metabonomics were uploaded into the **DETECTIVE** database and transferred into the central database of ToxBank.

Repeated dose exposure of doxorubicin induced arrhythmic beating and apoptosis in iPS cell derived cardiomyocytes. Transcriptomics data analysis shows that doxorubicin exposure even at lower concentration has affected various biological processes including cardiac muscle contraction, cellular ion homeostasis and energy metabolism. For example, genes regulating biological pathways like glycolysis and cardiac muscle contraction are down-regulated. After doxorubicin washout some genes previously found involved in cardiomyopathies retain abnormal expression. Metabolomics analysis shows doxorubicin induces metabolic alterations, especially related to disturbed mitochondrial metabolism.

DETECTIVE has generated novel metabolomics data defining endogenous metabolic response to a number of toxicants through integrated studies in cell models representing the heart, liver and kidney. A number of biomarker responses were identified based on these data that may be useful in identifying and classifying toxicity in these test systems.

iClusterPlus for an integrative clustering framework was used to integrate diverse '-omics' datasets in order to cluster samples allowing novel biomarker findings.

Integration of Biomarkers

One of the major goals of the **DETECTIVE** project is the identification of toxicity relevant biomarkers. To this end it is concentrating on bioinformatics and statistical analysis for identification of candidate biomarkers, which must be further selected and verified in on-going studies. The identification of biomarkers has combined two major strategies. A first strategy consists of the establishment of a biomarker repository based on data retrieved from relevant scientific literature. Furthermore, heterogeneous groups of biomarkers, including functional/cell type-specific read-outs and '-omics'-based read-outs, were identified for chemical-induced liver toxicity, in particular cholestasis and steatosis, renal toxicity and cardiotoxicity. These combined data were introduced into a biomarker repository that is now being used as a reference tool in the **DETECTIVE** project.

4.4.3 Selected Highlights: Renal Toxicity Assessment, *in vitro* Bile Canicular Architecture and BAC-GFP Pathway Reporter Platforms for Hepatotoxicity Assessment

Renal cell specific measurements and xCELLigence technology

Lactate production during cytotoxic stress of RPTEC/TERT1 cells: In **DETECTIVE**, the renal cell

system is based on the human telomerase-transfected proximal tubule cells RPTEC/TERT1 (*Wieser et al., 2008*). These cells form a stable contact inhibited monolayer that differentiates into a mature transporting epithelium in culture (*Aschauer et al., 2014*). In addition to these morphological changes, these cells also rely primarily on oxidative metabolism for their energy requirements once differentiated (*Aschauer et al., 2013*). We have previously shown that upon chemical injury RPTEC/TERT1 cells revert back to the highly glycolytic metabolism they sustain during their proliferation phase, and secrete the glucose metabolite lactate in high quantities (*Limonciel et al. 2011*). Thus in this project, we have used the measurement of lactate in cell culture supernatants as a marker of cellular stress indicative of cellular de-differentiation upon chemical exposure. In previous years, lactate measurements were used as one of the endpoints to choose test concentrations for '-omic' investigations, and to monitor the induction of cellular stress during repeated dose exposures. In the fourth year of the **DETECTIVE** project, lactate measurements were utilised to determine the optimal time for recovery between two series of repeat dose exposures to a reference compound A (*Figure 4.14*). The production and secretion of lactate within a 24h window was increased during the first treatment period and progressively decreased during the following 8 days to reach control levels, thus indicating a recovery from chemically induced injury and a return to normal metabolic function of these differentiated cells.

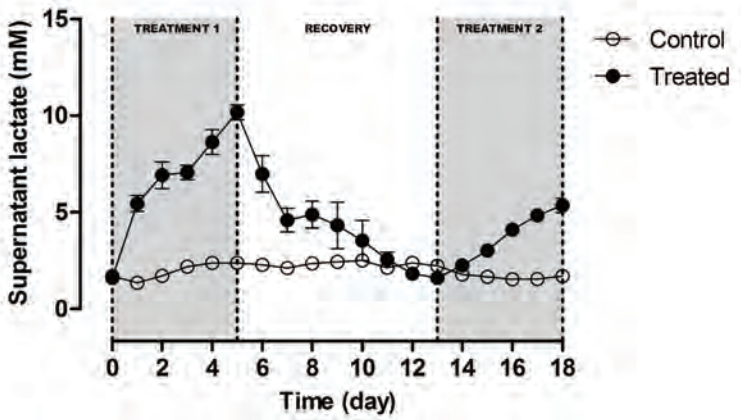


Figure 4.14 *Use of supernatant lactate as a monitoring endpoint for metabolic function in RPTEC/TERT1 cells. Exposure to a reference compound increases lactate levels during both treatments. Lactate monitoring indicated when the cells had recovered from injury after the first treatment. Results are mean ± SEM of the mM concentration of lactate accumulated within 24h in three biological replicates.*

xCELLigence impedance measurements: The xCELLigence impedance measurement system was also used to monitor RPTEC/TERT1 cell viability during short- and long-term

exposures to chemicals. In previous years, impedance measurements were used extensively, especially for dose range toxicity testing prior to '-omic' experiments in order to determine the most appropriate concentrations to use. In the fourth year of the **DETECTIVE** project, this system was employed to investigate whether two compounds of interest had synergistic or antagonistic effects on RPTEC/TERT1 cells death in dose range testing. *Figure 4.15* shows the monitoring of the impedance of RPTEC/TERT1 cells exposed to high concentrations of compounds B and C, either on their own, together with a low concentration of the other compound (co-incubation) or after a 24h pre-incubation with a low concentration of the other compound. The result on cell death (CI decrease) is a synergistic effect when low B and high C concentration are applied together, but a prevention of B-induced cell death when low C is given simultaneously. In both cases, a pre-incubation with a low concentration of the other compound appeared to reduce the effects of the test compound.

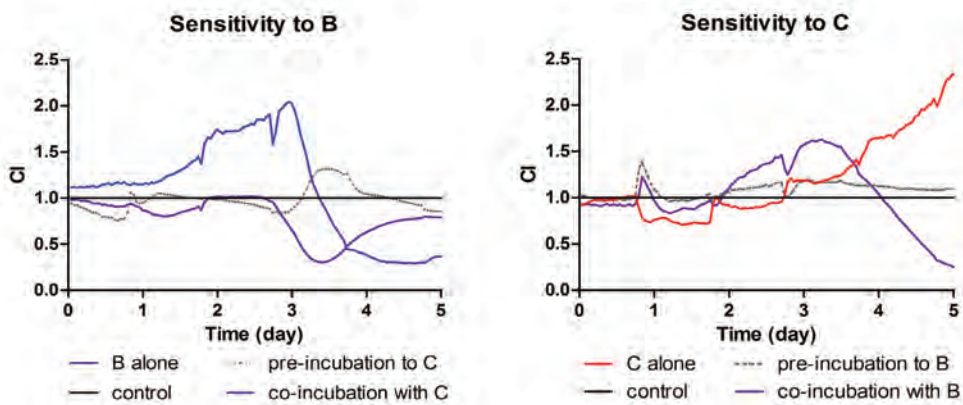
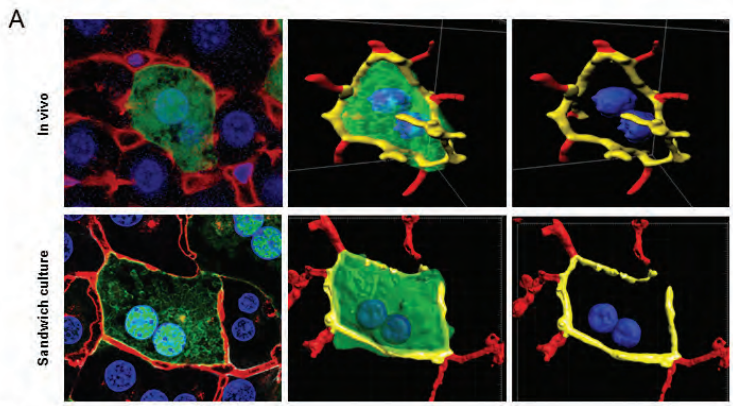


Figure 4.15 *Monitoring of RPTEC/TERT1 cells death upon exposure to compounds B and C. In the left panel, cells are exposed to a high concentration of compound B either alone (blue), together with a low concentration of compound C (purple) or after pre-incubation with a low concentration of C (grey). In the right panel, cells are exposed to a high concentration of compound C either alone (red), together with a low concentration of B (purple) or after pre-incubation with a low concentration of B (grey).*

Comparison of Bile Canalicular Architecture in Sandwich Culture and *in vivo*

In order to examine the degree of similarity between the collagen sandwich cultivation system and the *in vivo* situation we quantified hepatocyte morphology and bile canalicular structures. Distinction of individual hepatocytes was achieved with mice expressing GFP under the chicken beta-actin promoter, which gave a mosaic pattern of about 50% fluorescent hepatocytes. To assess the morphological structures, additional architectural immunostainings were carried out (*Hammad et al, 2014*). The hepatocytes were segmented by the cytosolic GFP signal, the bile

canalicular structure according to the signal of the antibody against the apical marker DPPIV, and the nuclei with the aid of DAPI staining (*Figure 4.16A*). Quantification of bile canalicular features in sandwich culture and *in vivo* showed striking similarities (*Figure 4.16B*). The overall length of bile canaliculi per hepatocyte was similar in cultured hepatocytes and *in vivo* (114 vs.139 μm/cell). Also the bile canalicular density per volume was similar (*Figure 4.16B*). Only the number of intersection nodes differed with lower numbers in culture. However, this difference is expected because bile canaliculi can only be formed at hepatocyte-hepatocyte interfaces and not in the direction of the collagen layer of the sandwich. In contrast, *in vivo* the third dimension is available for bile canalicular network extension and can therefore form more intersection nodes and have shorter branches in between intersections (*Figure 4.16B*). In conclusion, the bile canalicular network established by sandwich cultures is not identical to the *in vivo* situation due to the upper and lower collagen gels which prohibit extension of the canalicular network in this dimension. However, the bile canaliculi at the hepatocyte-hepatocyte interface show a high degree of similarity to the *in vivo* situation.



B

	Parameter	*Sandwich culture* (n=9)	*In vivo* (n=16)
Bile canaliculi	Length of BC network [µm/cell]	116.0 ±31.3	138.6 ±41.5
	BC density [m/mm^3]	19.50 ±7.86	15.45 ±3.65
	Intersection nodes per cell	3.89 ±0.78	14.69 ±4.71 *
	Length of intersection branch [µm]	19.18 ±6.97	7.23 ±1.32 *

* Significant difference $p<0.05$

Figure 4.16 *Quantification of hepatocytes and bile canalicular structures in vivo and in sandwich cultures (day 2). (A) Segmentation of morphological structures based on fluorescent images. Liver tissue (upper panel) and hepatocytes sandwich cultures (lower panel) from GFP expressing mice were immunostained for DPPIV and counterstained with DAPI. Segmented cells are displayed in green, nuclei in blue, bile canalicular ring of a hepatocyte in yellow and branching canaliculi in red. (B) Quantified parameters for the bile canalicular system in vivo compared to in sandwich cultures. * $p<0.05$ for the difference between sandwich cultures and in vivo.*

Basal bile canalicular dynamics in vitro: Bile canaliculi in sandwich cultures as described in the previous paragraph represent a mature state at day 2 in culture. However, on path to this stage bile canalicular morphogenesis represents a highly dynamic process. Initially, neighboring hepatocytes maximise their initial contact area approximately during the first 3 h after attachment (*Figure 4.17A*; stage 1). Within 6 h, a small lumen emerges at the cell-cell interface (*Figure 4.17B*; stage 2). Subsequently, the volume of this lumen is increasing and undergoes repeated cycles of contraction and dilation for 30 h (*Figure 4.17C*, D; stage 3). Finally, a relatively static mature bile canaliculus is formed (*Figure 4.17E*; stage 4, 36 h). In this period transport of large approximately 0.3 - 0.6 mm vesicles to the lumen can be observed (data not shown).

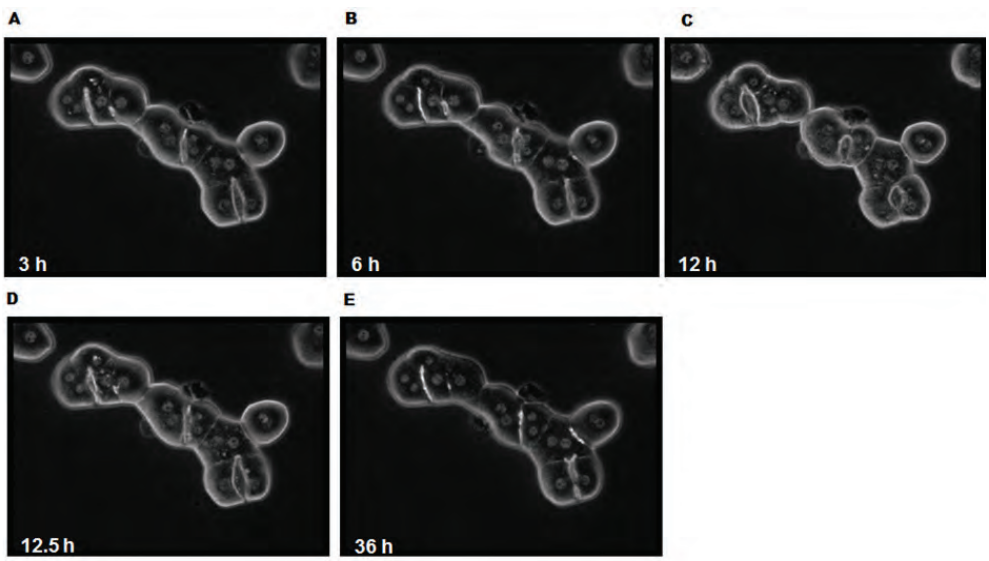


Figure 4.17 *Maturation process of hepatocytes in sandwich cultures. From plating on for about 4 h hepatocytes get in initial contact and maximise their interfaces - stage 1 (A). Afterwards, small lumina in the middle between two hepatocytes are arising – stage 2 (B). Between 10 and 24 h after plating this bile canalicular lumina are highly active and frequently start to arise (C) and collapse (D) – stage 3. When the hepatocytes are for more than 36 h in culture, these activities are getting into a calmer steady state (E) – stage 4.*

Functional bile canaliculi in vitro eliminates CMFDA similarly as in vivo: Stage 4 bile canaliculi (*Figure 4.17E*) have a much smaller luminal diameter compared to stage 3 (data not shown, preliminary quantification). A possible explanation of maturation from stage 3 to stage 4 is establishment of drainage to the culture medium which reduces intraluminal pressure.

To test this hypothesis we compared the bile canalicular secretion in sandwich culture and *in*

vivo using the cell marker CMFDA. CMFDA is taken up by the cells and turning fluorescent as soon as it is metabolized by the cell (it is a substrate for MRP2 present at the bile canalicular membrane) and eliminated via the bile. In sandwich cultures the green fluorescent CMFDA was observed in bile canaliculi ca. 5 min after addition of 1 μM CMFDA to the culture medium (*Figure 4.18A*). To compare the sandwich culture to the liver situation *in vivo* two-photon microscopy was applied (*Figure 4.18B*). Green fluorescence in bile canaliculi was observed 2 min after injection of 120 μg/kg CMFDA into the tail. This suggests that sandwich culture re-establish functional bile canaliculi and are capable of eliminating bile salts analogues similarly as liver *in vivo.*

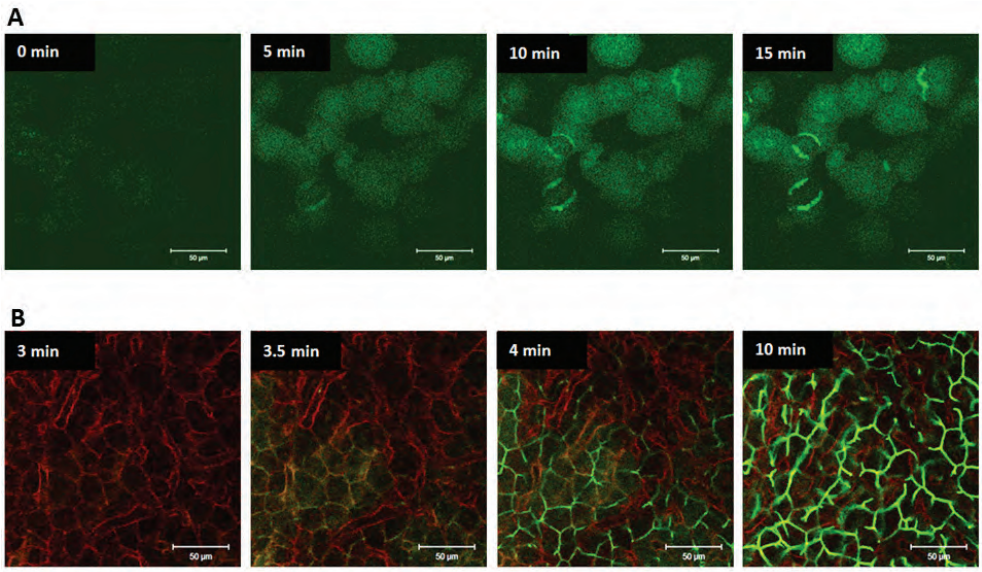


Figure 4.18 *Assessing functionality of hepatocytes in sandwich culture by monitoring bile salt transport. (A) In sandwich cultures the cell marker CMFDA (green) is up taken by the hepatocytes and its fluorescent metabolite excreted into the bile canaliculi. Metabolized CMFDA gets cleared into the luminal space. (B) When injected intravenously into mice CMFDA is activated (green) and eliminated very efficiently by the hepatocytes (red membrane). Already 2 min after injection CMFDA is mostly present in the bile canaliculi.*

Quantitative analysis of bile canaliculi structures in sandwich cultured hepatocytes: The robust identification of bile canaliculi by phase contrast microscopy allows a continuous monitoring of the bile canalicular lumen. For routine analyses of the influence of chemicals on their cholestatic properties it would be helpful to automatically quantify the bile canalicular lumen from time-lapse videos.

For this purpose we established an image analysis algorithm, which automatically finds and tracks the plasma membranes of the individual hepatocyte at the cell-cell interface. This

determination enables the quantification of the area of the enclosed lumen for each time frame. This approach relies on the analysis of the brightness difference in phase contrast, which appears brighter in the bile canalicular lumen than in the cytoplasm. In a first approach, we used primary mouse hepatocytes to establish the basic conditions for phase contrast-time lapse imaging of bile canaliculi. The procedure is now being used to assess alterations in canalicular structures on primary human hepatocytes.

The movie quantification process starts with the segmentation of each frame. Hereby the software automatically recognises hepatocytes and defines the endpoints of selected canaliculi (*Figure 4.19*, yellow asterisks). Typically, the analysis was performed in a frame where the canaliculi are small, since effects such as swelling are most clearly defined in those structures. The area of the canaliculi is calculated by the lines between the two endpoints (marked as the red area in *Figure 4.19*). The progression of the bile canaliculi size in each frame is then visualised as the green line *in Figure 4.19*.

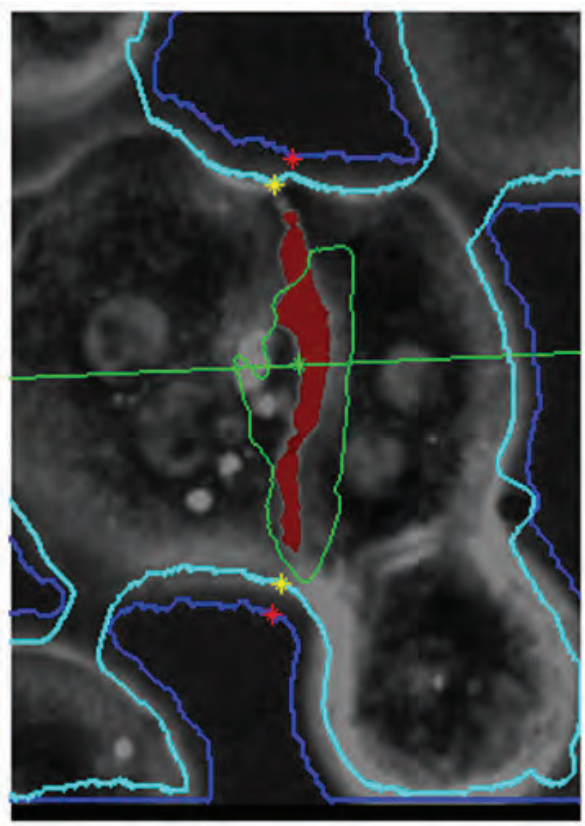


Figure 4.19 *Example of bile canaliculi segmentation in vitro for movie quantification. Segmented bile canaliculi showing its endpoints (yellow asterisks) and the segmented interface area (red) and the border of a previous step (green line).*

Crosschecking the segmentation process of the software showed that the recognition of the bile canaliculi in the movies had been precise. Moreover, the changes of area over time are very accurate (*Figure 4.20*).

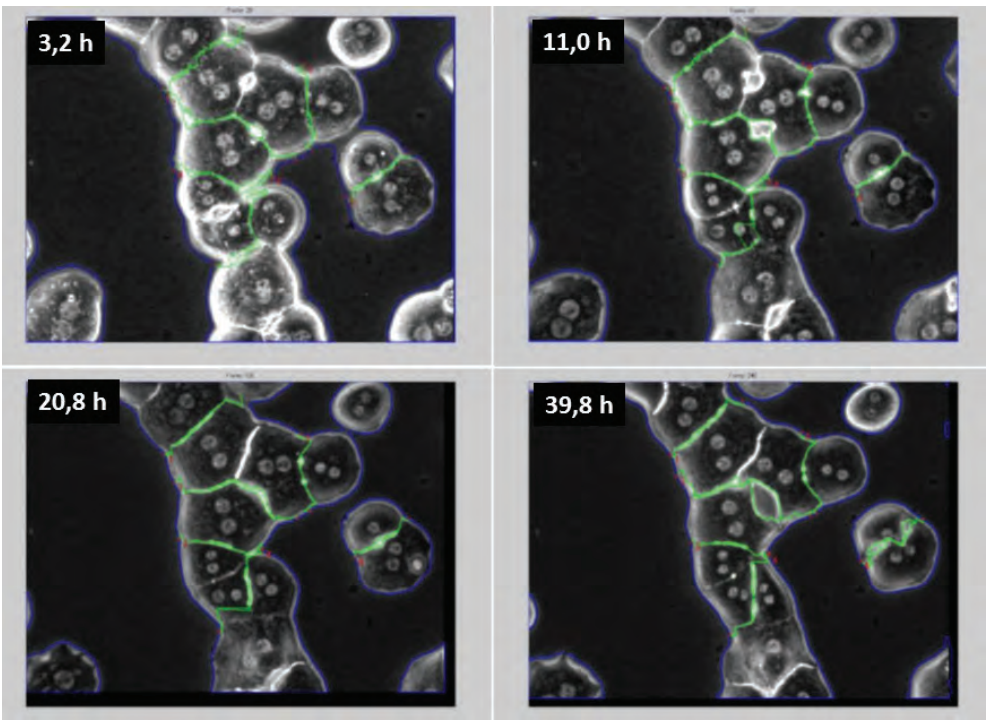


Figure 4.20 *Automated segmentation in snap shots out of a movie.*

An example of mature bile canaliculus showing morphological dynamics at day 3 is shown in *Figure 4.21A*. Frames correspond to 10min- interval of dilatation-contraction cycles for about 2-4 h which comprise the bile canaliculus over the entire hepatocyte surface. The images were then used to generate quantification software specifically designed to detect alterations in bile canalicular structures (*Figure 4.21B*). The quantification of the bile canalicular area illustrates a highly dynamic behaviour.

To validate the quality of the automated image analysis we manually evaluated two time-lapse videos with 500 and 450 frames, respectively. The areas obtained by the manual and automated technique showed a significant ($P < 0.001$) correlation with correlation coefficients of 0.9 X and 0.7 X, respectively (*Figure 4.21C*). Differences between the manual and automatic segmentations are not mainly due to clear errors from either method, but rather due to differences in interpretation of what constitutes the boundary.

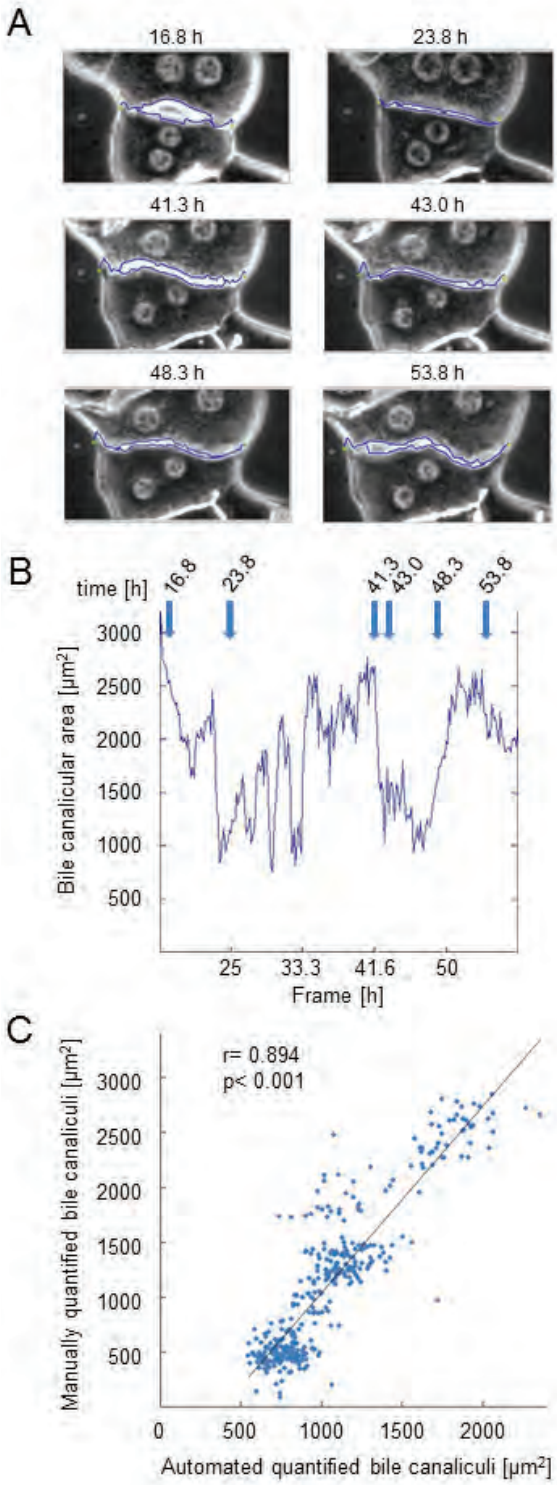


Figure 4.21 *Quantification of bile canalicular activities in sandwich cultures. (A) Morphological dynamics of hepatocytes in sandwich culture at stage 4 shown in still photographs taken from a time-lapse movie. (B) Quantifications of the luminal area over time between hepatocytes by image analysis are showing the activity of bile canaliculi during the recordings. (C) To validate the developed automated process, the quantifications were compared with manually measured luminal areas of each time frame. The correlation coefficients for different videos were between 0.9 and 0.7. The plot shows a correlation plot for one video.*

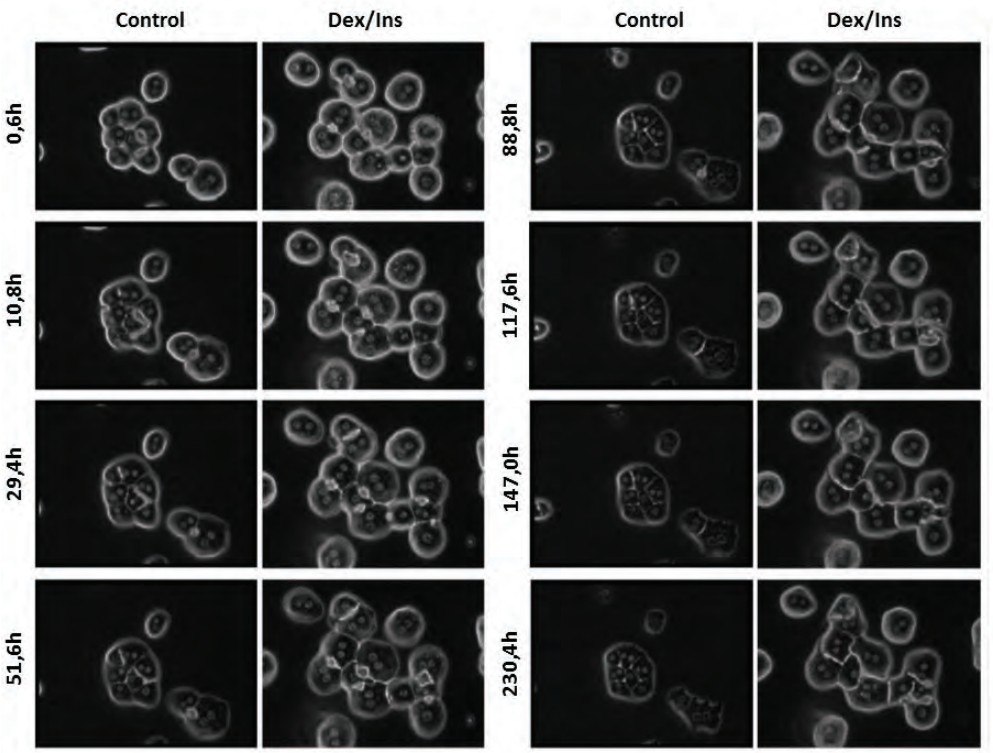


Figure 4.22 *Dexamethasone/insulin (Dex/ins) significantly increased the secretion of bile acids into bile canaliculi. Differences of bile canaliculi of control and dexamethasone/insulin treated hepatocytes after attachment between 0,6-230,4h in culture. Treated hepatocytes show a higher activity in bile acid secretion than the controls.*

The images were then used to generate quantification curves by using the software specifically designed to detect alterations in bile canalicular structures (*Figure 4.23*). Quantification of 21 canaliculi per condition resulted in larger bile canalicular areas for the dexamethasone plus insulin samples compared to controls (*Figure 4.23A-D*). The result shows an approximately 400 % increase of the bile canalicular area of the treated versus the control cells.

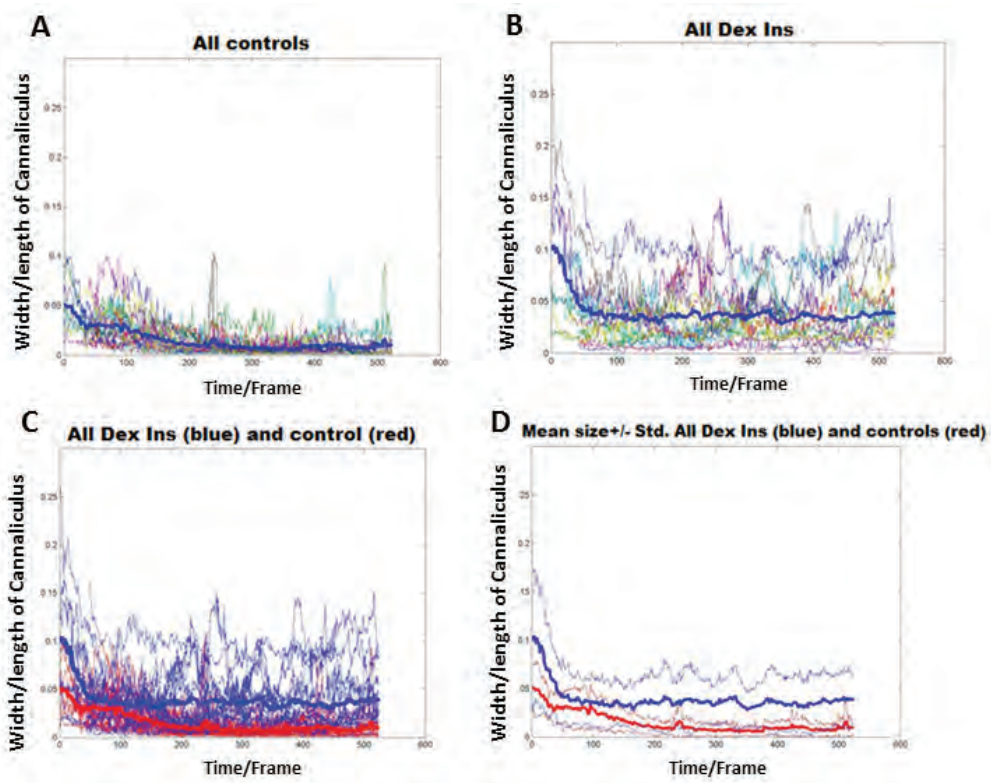


Figure 4.23 *Quantification of bile canalicular dynamics in control vs. dexamethasone/ insulin treated hepatocytes in sandwich culture. (A) Graphic representation of width/length of bile canaliculi of all control samples in a set of frames. Thick blue line represents mean values. (B) Graphic representation of width/length of bile canaliculi of all Dex/ins-treated samples in a set of frames. Thick blue line represents mean values. (C) Graphic representation of width/length of bile canaliculi of all control samples (red) and all Dex/ins-treated samples (blue) in a set of frames. (D) Graphic representation of the mean width/length of all control samples (thick red) and all Dex/ins-treated samples (thick blue) together with their respective standard deviation (light red and light blue, respectively) in a set of frames.*

BAC-GFP HepG2 Pathway Reporter Platform for Hepatotoxicity Assessment

Previously we have reported the generation of BAC-GFP reporter pathway of toxicity reporter platform based on BAC engineering of the human hepatoma HepG2 cells (*Figure 4.24*). This platform is based on well-described pathways involved in the adaptive stress responses of (liver) cells.

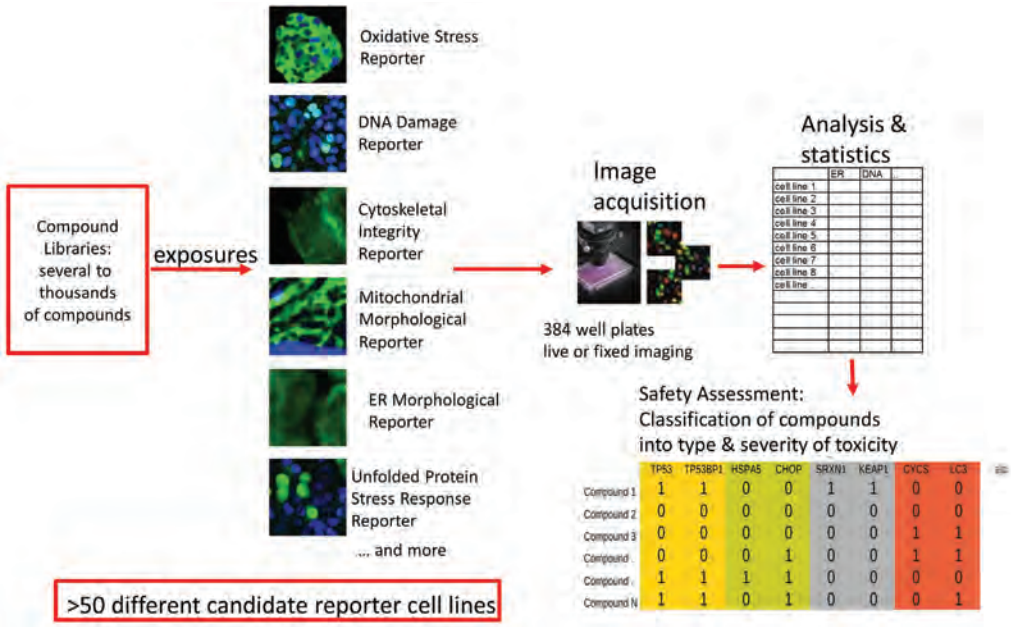


Figure 4.24 *Concept of the pathway of toxicity reporter platform.*

Key nodes of these pathways (i.e. the upstream 'sensors', transcription factors and downstream targets) are tagged with EGFP by BAC cloning technology (*Figure 4.25*). Using the BAC cloning technology to generate the reporters leads to a low overexpression and endogenous regulation of the tagged protein, in addition the original non-tagged target protein remains unchanged to ensure normal cellular function.

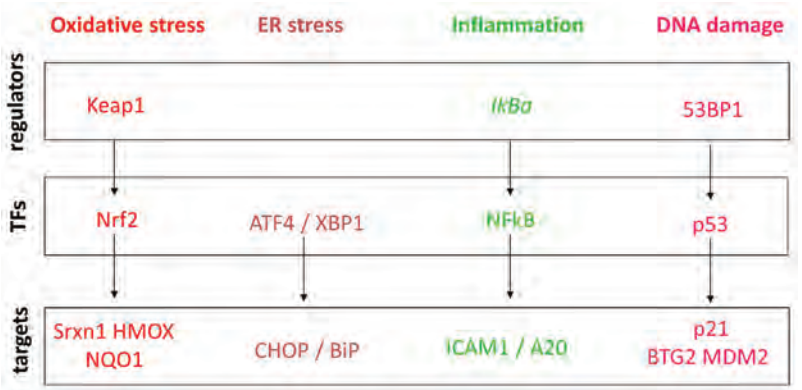


Figure 4.25 *Sensor, effector and targets within adaptive stress response pathways which are EGFP-tagged using BAC cloning technology and together constitute the cellular defense response to chemical insults.*

So far we tagged the key nodes from the Nrf2-regulated oxidative stress pathway (Keap1, Nrf2, Srxn1, HMOX, NQO2) , the ER-stress/unfolded protein reponse (UPR)-pathway (BiP, Chop, XBP1, ATF4), the DNA-damage-response pathway (TP53BP1, TP53, p21) and the NF-κB regulated anti-inflammatory pathway (NFKB1, RELA, ICAM1). *Figure 4.26* summarises several examples of our reporters.

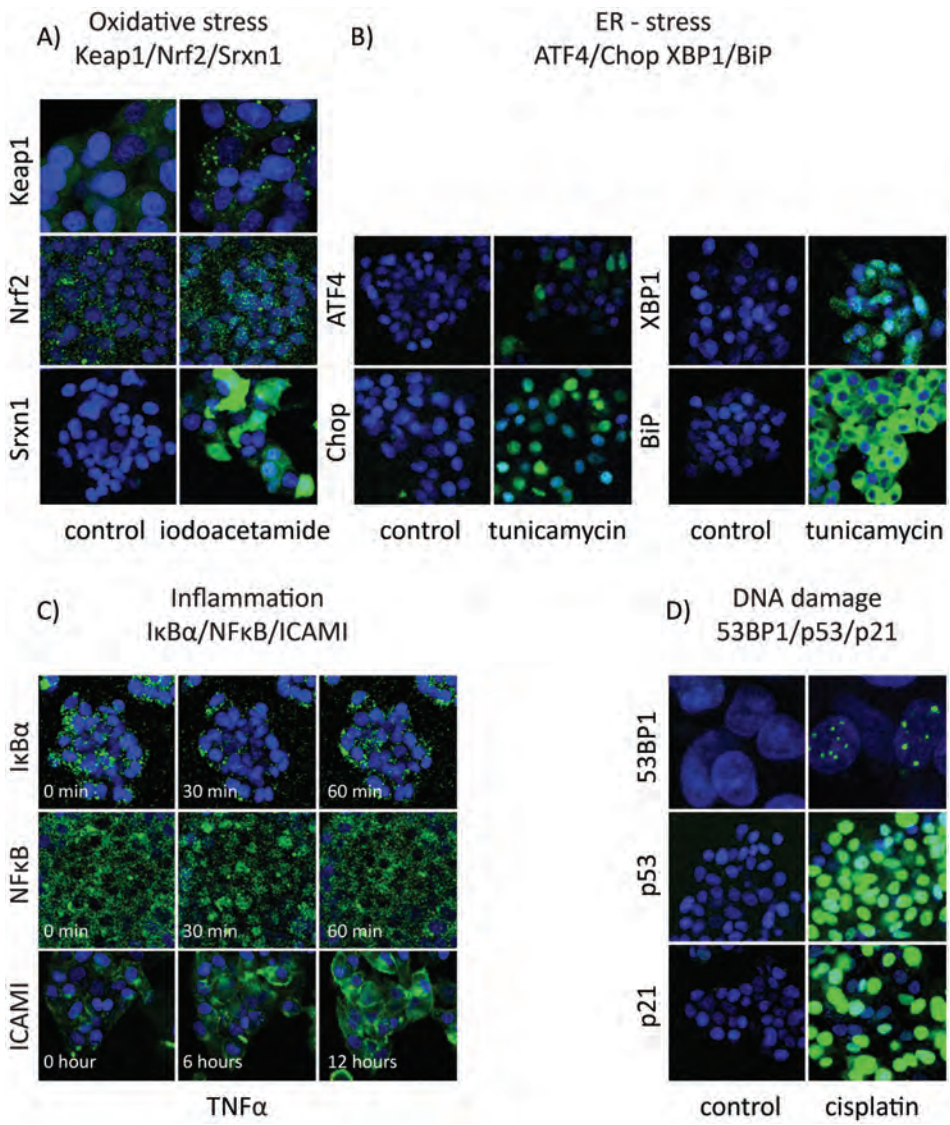


Figure 4.26 *Examples of BAC reporter cell lines of toxicity pathways in HCI. (A) Oxidative stress signaling. (B) ER-stress signaling. (C) Inflammation signaling. (D) DNA damage response. (Data soruce: Wink et al., 2014).*

In addition we have further complemented our platform in the past year with several new reporters. These reporters include other adaptive stress response pathways including the heat shock response (HSF1 and HSP72) as well as the metallothionein response (MT2). Moreover, these include several components that are closely related to the steatosis AOP which is part of one of the case study pillars within the **DETECTIVE** project (see section 3.4.3). These novel reporters include BSEP, OATP2, MRP2, PXR and FXR. All these reporters are currently being evaluated for functionality. In particular the steatosis biomarkers are in the process of being tested in a 3D matrigel environment with repeated dosing of steatosis-related compounds.

Image analysis pipeline: The simultaneous imaging capabilities of several high content live cell imagers in a screening laboratory easily leads to 100 GB of images (20,000–30,000 images) overnight. Therefore, automated high content image acquisition must be coupled to an integrated automated multiparameter-image analysis tool for fast and accurate quantification of the acquired images. We have established our own customised image analysis pipeline based on: ImageJ plugins, CellProfiler, HDF5, and quantitative data processing R-scripts (*Figure 4.27*; see also summary in *Wink et al., 2014*). Image loading, image metadata definitions, intensity and most morphological feature measurements, and the initial tracking of single cells is performed by standard CellProfiler modules. Raw images are loaded, and the metadata (i.e., well locations) is defined. After image processing (e.g., noise filtering), the nuclei are segmented using the nuclei-stained channel followed by single nuclei identification to enable the analysis of population distributions. These identified nuclei are used as seeds to detect the cell-boundaries using the GFP channel. Further image objects can be defined (e.g., foci, cytoplasm, and organelles). Single-cell tracking is usually included to enable single-cell based analysis over time. The segmentations are performed by a custom-made ImageJ plugin based on the watershed masked clustering algorithm; in addition, some morphological (e.g., skeleton) measurements are also performed by ImageJ plugins. These plugins have been integrated in the CellProfiler environment by making use of the python-javabridge utility provided by CellProfiler. The latest version of CellProfiler includes the option to store quantitative data output in a hierarchical data format: HDF5. An often overlooked aspect is data format standardisation for accessibility and interlaboratory data exchange. For this reason we chose to store the quantitative data in this format. Together with a plate layout text file, the quantitative data is analyzed and graphically displayed in an automated fashion using an in house developed R-package, which will be released in the public domain after publication. The automated analysis includes reorganisation and modification of the tracked objects and linkage of cellular features/phenotypes to cell mobility on the single cell level.

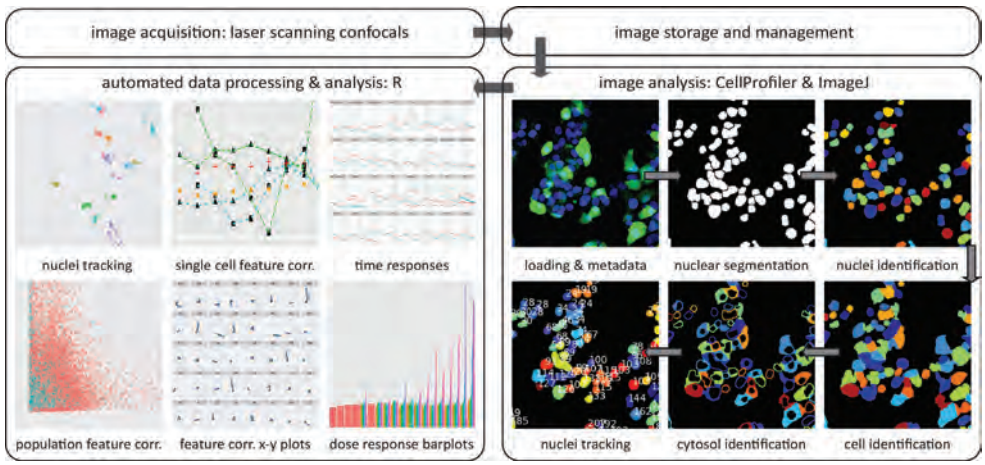


Figure 4.27 *Pipeline of high content imaging of BAC-GFP reporter cell lines. Image acquisition by laser scanning confocal microscopes is followed by storage in a central data storage utility including a database management system. Image analysis is performed using CellProfiler and integrated ImageJ plugins. Image analysis output (i.e., quantitative data) is stored in HDF5 files. R is used to interact with the data in HDF5 in an automated manner; several summary statistical output text files are generated, and in addition, a collection of plots to investigate the quantitative data are generated. Nuclei tracking: for quality control purposes of the tracking performance, the x and y-axes represent the x and y coordinates in the original images; single cell feature corr., single cells are followed in time (x-axis) for two selected measurements (y-axis), and the two selected measurements are, for example, cell speed and cytosolic intensity of the reporter cell lines. Time responses: the selected percentile of all the single-cell measurements (y-axis) in each well in the multiwell plate are plotted over time (x-axis). Population feature corr.: two selected measurements (x and y-axes) can be compared on a single cell basis on the entire plate for identifying dependencies (e.g., cell density and cytosolic intensity measurement). Feature corr. x-y plots: linear regression analysis for two selected measurements for each condition in the multiwell plate to identify correlations. Dose–response barplots: area under the time curve summary statistics of a selected measurement (y-axis) for each condition (x-axis) in the multiwell plate with increasing concentration (subparts x-axis). (from Wink et al. 2014).*

Application of reporter cell lines to monitor hepatotoxicity stress responses: In the past year we have used the BAC-GFP reporters to assess the time dynamics in stress response activity. We focused on the oxidative stress, UPR and DDR reporter lines. We first addressed the questions wether our reporters are selective for the respective pathway activation. For this purpose we have exposed all our BAC-GFP reporters to the model compounds that specifically induce oxidative stress, ER stress or DNA damage. The data indicate that our reporter cell lines are selective for the anticipated stress response (*Figure 4.28*).

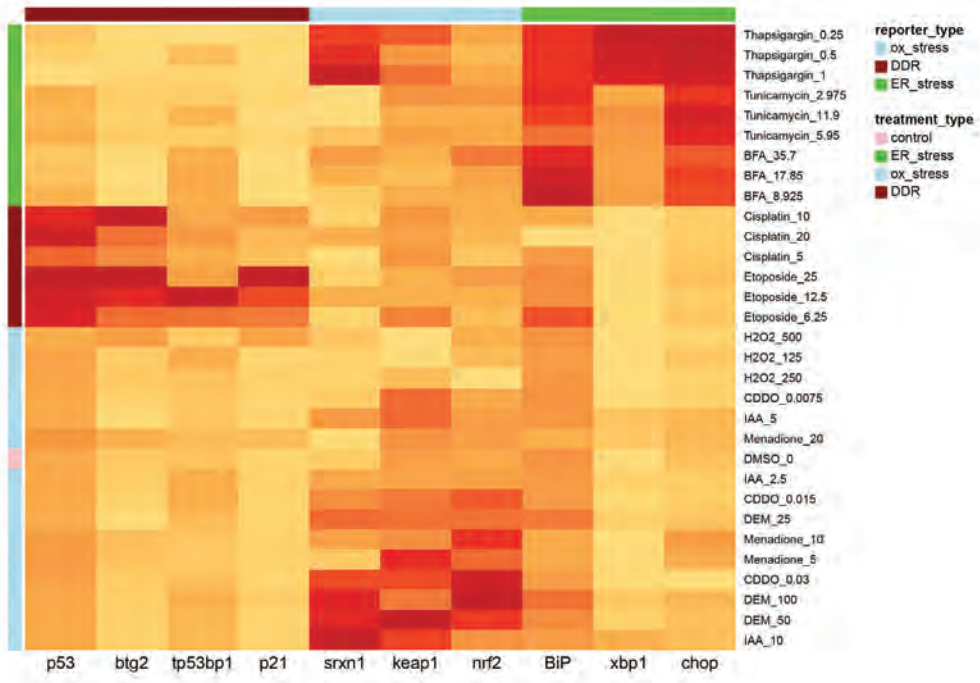


Figure 4.28 *Effect of selective pathway activitors on the activation of BAC-GFP HepG2 reporter cell lines. Note that model compounds that only activate e.g. ER stress/UPR cause the activation of ER stress/UPR reporter cell lines; also pro-oxidants primarily activate oxidative stress reporters, and DNA damage inducing compounds only activate the DDR reporters.*

As a next step we evaluated the response of our reporters to compounds that are known to cause hepatotoxicity. These compounds were selected based on the TG-GATEs data. For all TG-GATEs compounds we evaluated the relative expression of our downstream target gene expression, i.e. Srxn1, CHOP/DDIT3, and p21. For each downstream target we identified the top 10 compounds that would induce these target genes in primary human hepatocytes. Then we exposed our reporters to these 10 compounds followed by live cell imaging of the reporters and quantitative analysis of the response. These data indicated that our reporters can pick up the stress responses that are observed in primary human hepatocytes, indicating a translation of stress responses from PHH to our HepG2 reporter cell model.

Next we exposed our reporter cell lines to a larger panel of compounds. These have included the **SEURAT-1** standard reference compound list. We have exposed our GFP-Srxn1-HepG2, GFP-CHOP-HepG2 and GFP-p21-HepG2 reporter cell lines to different concentration of the selected compounds. This list of compounds includes: acetaminophen, aflatoxin B1, allyl alcohol, amiodarone, bosentan, CCl_4, chlorpromazine, DMNQ, doxorubicin, tamoxifen, valproic

acid, FCCP, fluoxetine, iodoacetamide, methotrexate, oligomycin A, rifampicin, rotenone, TO901317. Several compounds are expected to induce oxidative stress (iodoacetamide), or DNA damage (aflatoxin B1). Indeed we could determine that the **SEURAT-1** standard reference compounds induced the anticipated responses in our reporter cell lines (*Figure 4.29*). Interestingly, in general compounds that do affect the mitochondria were strong inducers of the Srxn1 response, including FCCP, rotenone, oligomycin. This raises the question whether other inducers of the Srxn1 response would also induce mitochondrial toxicity.

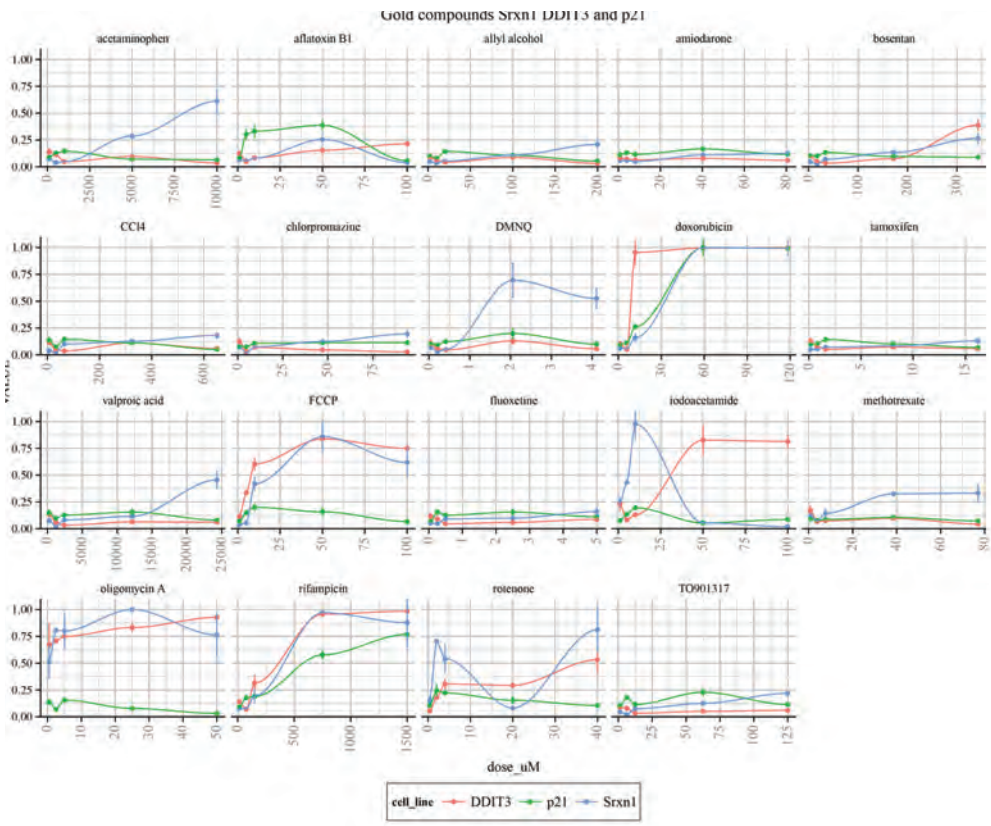


Figure 4.29 *Effect of* ***SEURAT-1*** *standard reference compounds on BAC-GFP reporter activation. GFP-Srxn1, GFP-CHOP and GFP-p21 reporter cell lines were treated with the various reference compounds at the indicated concentrations followed by 24 hr live cell imaging of reporter induction. The percentage of cells (VALUE) that showed a GFP intensity 3X above the average control cell level were quantified. The average effect of at least two independent experiments is shown.*

Currently we are expanding this work and are investigating the effect of ~150 DILI compounds on the different reporters. All exposures have been performed and we are currently analysing the data.

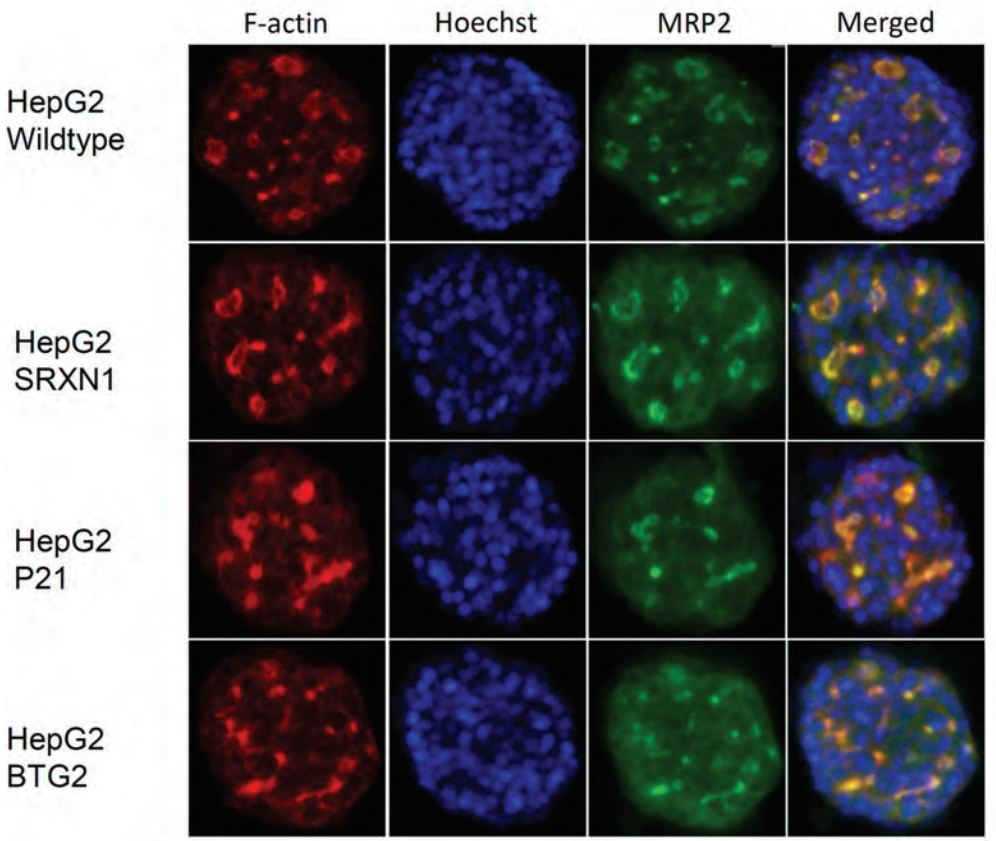


Figure 4.30 *Differentiaton capacity of different BAC-GFP HepG2 reporters in 3D spheroids. Reporter cell lines were cultured for 4 weeks in hydrogel followed by fixation and immunofluorescent staining for the indicated markers. Note that the differentiation was similar to wt HepG2 cells.*

Next we evaluated whether our reporter spheroid systems would also respond to the model compounds and could be analysed in a 3D context. Importantly, the GFP-Srxn1-HepG2 spheroids were responsive to diethylmaleate; the GPF-CHOP-HepG2 spheroids were responsive to thapsigargin, and the GFP-p21 spheroids were responsive to aflatoxin B1 (*Figure 4.31*).

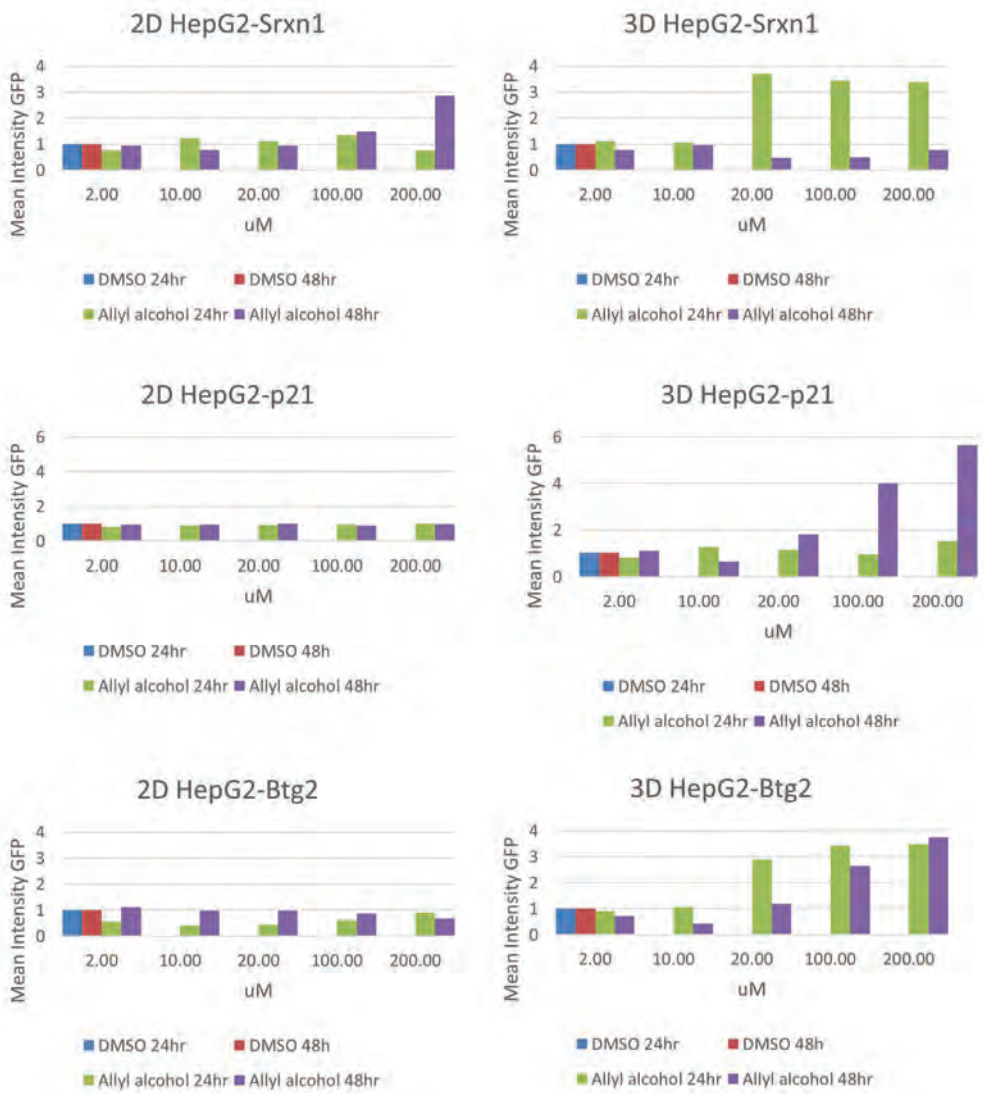


Figure 4.31 *Functionality of 3D HepG2 reporters for induction of stress responses. Different reporter cell lines were cultured for 4 weeks in hydrogel followed by treatment for 48 hr with the indicated positive control compounds to induce reporter activation.*

Finally we evaluated whether our spheroid systems would pick up responses that are not present in 2D HepG2 reporter systems. Since the HepG2 reporters have increased metabolic capacity, we selected a model compound that requires bioactivation for its cytotoxicity, the **SEURAT-1** standard reference compound allyl alcohol. Allyl alcohol did not induce much stress response activation in 2D HepG2 cells. Interestingly, when 3D HepG2 spheroids were

treated with allyl alcohol, we observed strong activation of the stress reporter cell models (*Figure 4.32*). This indicates that the 3D spheroid reporters can be used as a next tier in the safety evaluation of chemicals.

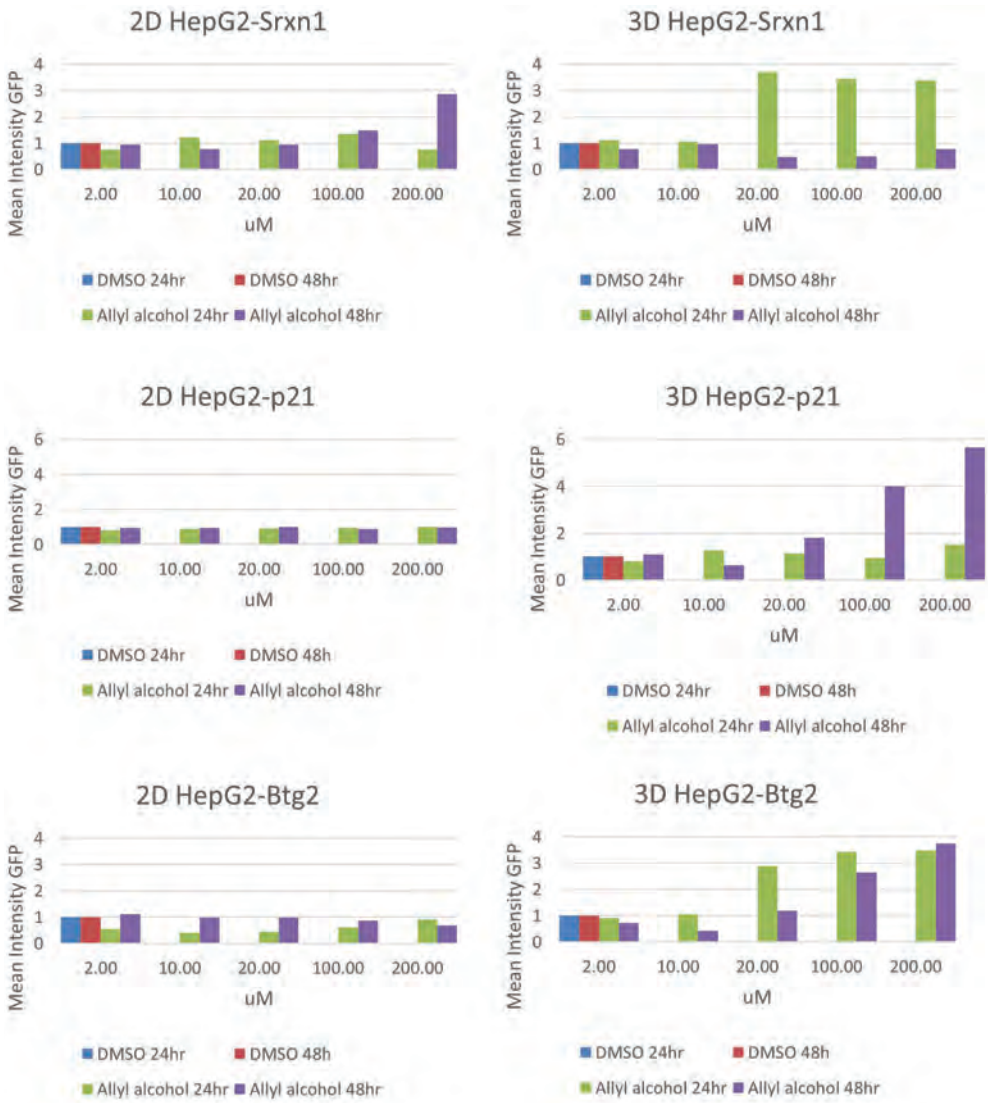


Figure 4.32 *Effect of allyl alcohol on reporter activation in 2D and 3D HepG2 cultures. Indicated HepG2 reporter cell lines were cultured in 2D or as four week 3D spheroids followed by treatment with the indicated concentration of allyl alcohol for 24 hr (green bars) or 48 hr (black bars). GFP intensity was calculated after automated imaging. Note that allyl alcohol is mildly affected Srxn1-GFP at 48 hr, while strongly affected all other reporters in 3D spheroid cultures.*

4.4.4 Innovation

The human proximal tubule cells line RPTEC/TERT1 cells, which have been modified to over express the enzyme telomerase represent the DETECTIVE renal cell system. These cells share many of the characteristics of the proximal tubule in vivo, but do not undergo replicative senescence so can be expanded practically unlimitedly. RPTEC/TERT1 when differentiate have high oxidative capacity and low glycolytic requirements. Chemical induced stress often causes an energy crisis in cells through to example mitochondrial injury. We have shown that RPTEC/TERT1 increase glycolytic capacity when exposed to many chemicals and that this can be simply measured as increased lactate in the supernatant medium. Since this is a non-invasive procedure lactate measurement is an excellent method for measuring proximal tubule injury in real time.

Another significant achievement of the **DETECTIVE** project was the further development of *in vitro* liver models allowing the formation of proper bile canalicular architecture in the **DETECTIVE** hepatocyte cell systems. Side-by-side morphological and histochemical comparison between the **DETECTIVE** *in vitro* cell system and *in vivo* models has demonstrated a high degree of similarity of the bile canaliculi at the hepatocyte-hepatocyte interface. Therefore the hepatocyte cell system established in **DETECTIVE** is more accurately reflecting the *in vivo* situation to be exploited in toxicity screening. Moreover, we have proposed that the bile canalicular size and dynamics could represent novel biomarkers predicting cholestasis in *in vitro* compound screening.

Previously we have reported the generation of a BAC-GFP reporter platform of toxicity pathways based on BAC engineering of the human hepatoma HepG2 cells. The wide range of the sensor cell lines have been demonstrated as an invaluable tool for efficient and sensitive determination of related toxicity pathways. Yet, the range of the sensor cell lines has been extended including reporters of BSEP, OATP2, MRP2, PXR and FXR monitoring, which represent stress pathways that are closely related to the manifestation of the steatosis AOP.

4.4.5 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.

In total **DETECTIVE** has been actively engaged in four of the six **SEURAT-1** Working Groups (see section 4.10.3). Partners of the **DETECTIVE** consortium are co-leaders in the Data Analysis Working Group, the Gold Compounds Working Group, the Stem Cells Working Group and the Mode-of-Action Working Group. When considering the selection of standard reference compounds in the consortium, **DETECTIVE** has always consulted ToxBank for their expert advice. **DETECTIVE** partners 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. The massive datasets generated by the **DETECTIVE** partners have been converted to ISATAB format and submitted to the ToxBank Data Warehouse for storage. **DETECTIVE** has concentrated its efforts on providing effective means of data collection and storage.

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. **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 (see section 3.2).

4.4.6 Expected Progress within the Fifth Year

In the final year, **DETECTIVE** will focus on verification of novel sets of biomarkers with predictive value for organ-specific and general toxicity, including chronic toxicity exposure scenario in a course of case studies and novel experiments to be performed with additional sets of carefully chosen compounds. The organ-specific and generic biomarker sets have been revealed after integrated data analysis of both own and publically available data-rich '-omics' datasets. The data verification will include both '-omics' and high-throughput imaging experiments. The main aim is the determination of organ-specific and general biomarkers or adverse pathways with chronic toxicity predictive values.

4.4.7 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 pluripotent 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 future research initiatives, along with testing and assessing several other human cell systems and establishing high-throughput screening platforms for various drug libraries. An Additional aspect to be addressed is the development of more physiologically relevant 3D dynamic flow systems and further systems miniaturisations for increasing high-throughput capacities and reduced assay costs.

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Awards

Limonciel, A. (2013): Lush-Prize, Young Researcher Award 2013 for her research into the improvement of *in vitro* models for testing toxicity effects on human kidneys.

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

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4.5.1 Introduction and Objectives

There is a desire to be able to obtain information regarding the safety of a cosmetic ingredient or impurity 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 Kinetic (PBK) models).

However, prediction of repeated dose toxicity poses a real challenge to computational modelling to provide a viable alternative to animal testing. Consequently, *in silico* models have previously been considered too simplistic to model the complex interactions underlying chronic toxicity and the expectation of a single computational approach to predict these is limited. Moreover, there is a well-recognised paucity of toxicological data and in particular a lack of openly available databases. These needs are being put in the context of computational modelling being a key focus in 21st Century Toxicology and the needs and requirements for a modern toxicological science.

The knowledge gaps are illustrated and summarised in *Figure 4.33*.

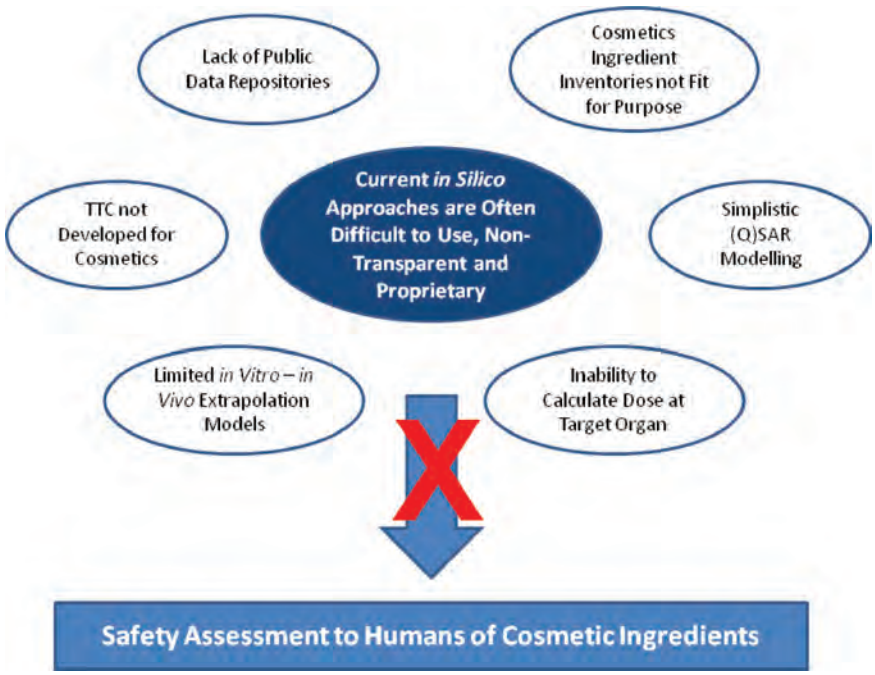

Figure 4.33 *Summary of the knowledge gaps preventing the assessment of the safety of cosmetic ingredients to humans from computational techniques.*

Therefore, the aim of the **COSMOS** project is to develop tools for the retrieval and supporting the prediction of repeated dose toxicity to humans for cosmetics-related chemicals, the integrated use of multiple models being expected to provide an alternative assessment strategy. **COSMOS** is at the centre of efforts to integrate reliable and open access toxicity data, TTC, grouping for read-across, (Q)SARs and modelling of biokinetics with the opportunities offered by informatics and the toxicity pathway approach.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). The workflows developed 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 toxicological data with an emphasis on repeated dose exposure;
- Create an inventory of known cosmetic ingredients and 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 (Q)SARs 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 kinetic (PBK) models to predict target organ concentrations and extrapolate from *in vitro* to *in vivo* exposure scenarios;

➠ Use KNIME technology to integrate access to data and modelling approaches into adaptable and flexible computational workflows that will be publicly accessible, providing transparent methods for use in safety assessment of cosmetics.

Later in this chapter, work undertaken and results achieved in implementing the models developed within **COSMOS**, e.g. PBK, into KNIME and making them publicly accessible via the KNIME WebPortal together with documentation and user guidance in COSMOS Space will be highlighted as major achievements of the fourth project year (see section 4.5.3).

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 Deliverables 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, both to the cross-cluster Level 3 case study scenarios on read-across as well as to the *ab initio* case study for chemical selection.. COSMOS DB version 1.0 was released publicly in December 2013.

Key Deliverables 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 data sets.

Key Deliverables 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 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 (MoA) knowledge.

Key Deliverables 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 kinetics modelling (e.g. through physiologically-based kinetic (PBK) models); a better understanding of the effect of the test system (e.g. sorption) and chemicals (e.g. volatility, stability) properties relating to extrapolation; and modelling and prediction of metabolism. These models can be used to determine the internal exposure (dose at target organ level) necessary to elicit the effect. **COSMOS** will thus help to apply highly targeted assays within **SEURAT-1** that could be developed and used to provide evidence to support the **SEURAT-1** knowledge of pathways, within the *ab initio* case study.

4.5.2 Main Achievements and Challenges in the Fourth Year

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

COSMOS Database

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 40,000 unique structures are flexibly searchable by name, CAS number, graphical representation, SMILES strings or other identifiers. COSMOS DB version 1.0 was made publicly available in December 2013 from the URL http://cosmosdb.cosmostox.eu. A webinar was held explaining use and application of the database, the recording and a short user guidance are available together with a summary of the DB content from the **COSMOS** website (http://www.cosmostox.eu/what/COSMOSdb).

In 2014, several important developments supporting the inter-**SEURAT-1** data exchange and the creation of the **COSMOS** No Observable Adverse Effect Levels (NOAEL) and TTC databases were made. To further support the interoperability, COSMOS DB has been enhanced with an application program interface (API) which is built around the OpenTox standards. After extensive testing the API has been successfully put in production and now enables the automated integration between COSMOS DB and ToxBank. ToxBank seamlessly retrieves and displays information from COSMOS DB thus facilitating the use of the public **SEURAT-1** data around the cluster. COSMOS DB provides ToxBank with detailed chemical information, including chemical name, composition type, material type, molecular formula and depiction of the chemical structure, if any. In addition, a summary of the toxicological data associated with the chemical of interest is provided, including the type and number of toxicological studies available, which are organised by their original source, facilitating an intuitive presentation to the end user. The API provides a link to the COSMOS DB page for the chemical of interest, to allow the user to explore the available data in detail. The COSMOS DB API was further extended to provide the available toxicological numeric endpoints – Highest No Effect Level, Highest Effect Level, etc – in addition to the summary of the available toxicological data for all COSMOS DB studies originating from the US FDA PAFA database.

COSMOS DB is supported by COSMOS Space (http://cosmosspace.cosmostox.eu) which facilitates sharing of predictive toxicology resources (data sets, models, workflows, documentation, meta-data) and which links to COSMOS DB and COSMOS KNIME workflows. The architecture was updated, functions optimised, new interfaces implemented, including for the interaction with COSMOS KNIME DB, and middleware checked for reliability and security. The previous COSMOS Space Wiki facility has been successfully replaced with an in-house developed facility solving initial collisions of credentials.

Threshold of Toxicological Concern (TTC) Approach for Cosmetic Substances

Progress was made towards the completion of the Threshold of Toxicological Concern (TTC) work with regard to the COSMOS TTC dataset and development of the oral-to-dermal extrapolation approach. Specifically with regard to the TTC data, a number of activities relating to the toxicity data were undertaken. This included the planning and execution of two different rounds of Quality Control through ILSI-EU COSMOS Expert Group 1. These decisions were presented to Cosmetics Europe and have allowed the COSMOS TTC NO(A)EL dataset to being finalised. Further, the utility of the Cramer Classes (*Cramer et al.,1978*), as implemented in ToxTree, was evaluated by manually assessing allocation of compounds to the various classes and comparison with ToxTree predictions. A number of recommendations to improve the predictive capability of ToxTree have been made and subsequently implemented into the software. As a result a strategy was established to be able to apply the Cramer Classes successfully to the COSMOS TTC dataset.

The COSMOS DB data model has been revisited and updated to host the COSMOS NOAEL and TTC databases. The updates allow the storage and display of N/LOAEL values associated with a chemical and/or toxicological study as well as the extraction of TTC datasets from the COSMOS NOAEL DB. N/LOAEL values are stored together with all relevant metadata including, but not limited to, the source of the value and the regulatory body which has determined that endpoint particular value. The COSMOS TTC database will host the TTC values as determined by the ILSI-EU COSMOS Expert Group and will be available after the final evaluation of the data.

The oral-to-dermal extrapolation approach relevant to TTC was completed by ILSI-EU Expert Group 2. The approach includes a decision tree with various steps to evaluate whether the TTC methodology can be applied to dermal exposure, with an assessment (through calculation) of the relative skin permeability of compounds; the skin permeability effort has been supported by collation of data and the redevelopment of models for the calculation of permeability coefficients and maximal flux. A number of case studies for cosmetic-relevant materials have been developed to illustrate the applicability of the decision tree. The algorithms and skin permeability models were coded into KNIME workflows.

The COSMOS TTC work has been presented in the ASCCT hosted **COSMOS** webinar 'Threshold of Toxicological Concern – an approach for safety assessment and its applicability to cosmetics-related chemicals' on 24 July 2014, the slides and a recording are available through the COSMOS website (http://www.cosmostox.eu/what/ttc).

Computational Tools for Toxicity Prediction

In order to develop an *in silico* strategy for the evaluation of potential binding to, and possibly potential activation of, nuclear receptors involved in the development of liver steatosis (e.g., LXR and PPARγ), a number of computational approaches have been applied and integrated. These methodologies include: molecular modelling (e.g., docking, pharmacophore search, fingerprint-based similarity), QSARs (quantitative structure-activity relationships) and structural alerts. This developed *in silico* strategy was proposed as a Level 2 **SEURAT-1** case study and is intended to support the Mode-of-Action/AOP framework for toxicity prediction.

To support bioavailability estimation after oral and dermal exposure, several scenarios for use of NOAEL data have been proposed and *in silico* models predicting gastrointestinal absorption (GIA) and skin permeability have been developed. For the estimation of GIA, permeability constants obtained from double sink parallel artificial membrane permeation assay (PAMPA) were used and a multiple linear regression (MLR) model was developed as an improvement of the model reported by *Nakao et al. (2009)*. For the estimation of skin permeability, coefficients obtained in human skin *in vitro* assay(s) were used and a MLR model was developed as an improvement of the model by *Potts & Guy (1992)*.

Additionally, several KNIME workflows were developed to support *in silico* modelling including analysis of the chemical space using Principal Component Analysis (PCA) on various descriptors and functional groups profiling. Furthermore a profiler for the prediction of potential binding to nuclear receptors related to hepatosteatosis was developed based on structural fragments and physicochemical features identified as essential for the binding to the receptors; as well as a profiler for mitochondrial toxicity, the grouping approach to determine the structural alerts being developed with an emphasis on hair dyes.

The workflows are freely accessible and executed in a web browser via the COSMOS KNIME WebPortal (http://knimewebportal.cosmostox.eu), with registration through COSMOS Space (http://cosmosspace.cosmostox.eu), where detailed descriptions of the available workflows can also be found. No installation of software is necessary.

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 Assay (VCA), implemented in the open-source KNIME platform. It is designed to model *in vitro* assays and takes into account the chemical's fate in the *in vitro* test system, the cell dynamics, and predicts viability and mitochondrial membrane potential disruption. It has been parameterised for hepatocytes (HepaRG and HepG2 in particular) and cardiomyocytes.

In order to support *in vitro* to *in vivo* and route-to-route (oral to dermal and inhalation to dermal) extrapolations, several physiologically-based toxicokinetic (PBTK) models were calibrated for cosmetic ingredients and drugs. Those PBTK models (also implemented as KNIME workflows) take account of uptake in different tissues (gastrointestinal tract, skin, lungs) and a methodology to calibrate them without animal testing has been developed. PBTK models have been coupled with the VCA models to enable realistic estimates of *in vivo* effects from *in vitro* data. In a further step toward integrated multi-scale modeling, a 2D liver model including mechanisms for cell necrosis and cell proliferation has been coupled to the PBK models. This allowed for the analysis of the effect of the accumulation of compounds on hepatocyte viability and detoxification capacity after long-term repeated exposure.

Other tools developed include a model for human bioconcentration and QSAR models to predict hepatic clearance. Several of the biokinetic models have already been publicly released into the COSMOS KNIME WebPortal.

COSMOS KNIME Software and Workflows

In order to support the implementation and dissemination of the computational models developed in the **COSMOS** project, the KNIME Server as well as the KNIME Analytics

Platform desktop application have been extended and improved in many ways during the last year. Quite a few of those extensions were triggered by the requirements inside **COSMOS**, such as extended database support with many additional nodes, a new integration of the R scripting language, or the ability to write to almost arbitrary location with writer nodes. The KNIME Server client now allows to open and edit workflows hosted on a KNIME Server via a simple double-click which saves the user from explicitly down- and uploading workflows.

The COSMOS KNIME WebPortal has been extended. The version launched publicly for SOT 2015 (http://knimewebportal.cosmostox.eu) includes biokinetic simulations using PBK models, models for the Virtual Cell Assay and human bioaccumulation factor, prediction of skin and oral (PAMPA) absorption, or profiling for potential nuclear recptor binding. More workflows will be made available during the fifth project year. In addition, a common format has been developed for all workflows for documentation and guidance, available through COSMOS Space (http://cosmosspace.cosmostox.eu).

4.5.3 Selected Highlight: Implementation of the COSMOS Models into KNIME Workflows with WebPortal Versions for Easy-to-Use Access

Introduction and State of the Art

The models developed within the **COSMOS** project were implemented into KNIME workflows to make them easily accessible for the end-users. In particular, they were also adapted into specific versions for the COSMOS KNIME WebPortal, which allows a user-friendly execution of the models through a web browser, without installation of any software.

KNIME (www.knime.org) is the modular platform which integrates access to chemical data, data processing and analysis, modelling approaches, profiling of structures and calculation of properties in a flexible way. By means of graphical workflows, data are read from various data sources and subsequently transformed into suitable formats for model building and/or visual analysis. KNIME provides a simple extension Application Programming Interface (API) which allows for easy integration of new methods which are usually represented by so-called *nodes*. Since KNIME is open source it is a suitable platform for developing and deploying the computational methods that are being developed in the different **COSMOS** work areas. The flexible computational workflows are adaptable and form a set of building blocks allowing users to incorporate their own data and approaches, where applicable.

KNIME is a desktop program which runs locally on a computer and uses a directory on that computer to store the workflows. In context of the **COSMOS** project, it was desirable to easily share the workflows for all working groups during development. This is, in principle, possible using a shared folder but in practice slow network connections and different authentication and permission systems render this approach impractical. Moreover, once finalised, the workflows

should be made easily accessible to the public and usable even for non-experts in KNIME. In addition, functionality was envisaged to archive different versions of a workflow during development and to retrieve them if needed. In this way, experiments, i.e. model calculations, carried out previously, could be reproduced even when the underlying workflow has changed over time. Therefore the extension of the KNIME Server including a versioning infrastructure was a major area of work.

The overall aim was to make **COSMOS** computational workflows implemented in KNIME publicly accessible and provide transparent, flexible and easy to use methods to support the safety assessment of cosmetics-related chemicals.

Approach

The KNIME integration platform features required for the work in **COSMOS** were addressed successively with updates of the KNIME Server and KNIME extensions to allow additional data sources and tools to be integrated, by means of (i) implementation of new nodes; (ii) setup of an archival framework allowing reproducible execution of workflows; (iii) allowing sharing of methods and completing workflows via a central repository; (iv) providing workflows to end-users via a simple (web-based) frontend.

The solution to the issues described above was the definition of a clear and easy to use interface for extensions and the introduction of a central KNIME server. The server not only hosts a repository of workflows but also offers browser-based access to pre-configured workflows.

Furthermore, specific questions and functionalities were addressed when needed for the implementation of the **COSMOS** models into KNIME workflows and adapting them for the WebPortal versions.

Results

New developments relating to the KNIME platform. The KNIME Server has been implemented which additionally includes an extension to the KNIME desktop allowing access to the server (essentially the workflow repository). The desktop side of the KNIME Server is represented by the so-called KNIME Explorer, which is a view inside the graphical user interface that shows the different workflow repositories, e.g. the local workspace, and the COSMOS KNIME server (see *Figure 4.34, left*). Through the KNIME Explorer, workflows can be up- and downloaded to and from the server, they can be executed on the server, and the results can be retrieved afterwards. Moreover, fine-grained read, write, and execute permissions for the workflows can be configured through the Explorer. A central server for the **COSMOS** project has been set up for all **COSMOS** partners to share workflows.

The server part of the KNIME Server handles the requests from the KNIME desktop clients, but also provides a web portal where pre-configured workflows can be executed from within a web browser, in this case the COSMOS KNIME WebPortal to make **COSMOS** models publicly accessible and executable. *Figure 4.34* (right) shows an example with the WebPortal server repository containing all available workflows in the left part. The right part shows the execution of the selected workflow (input of the query chemical).

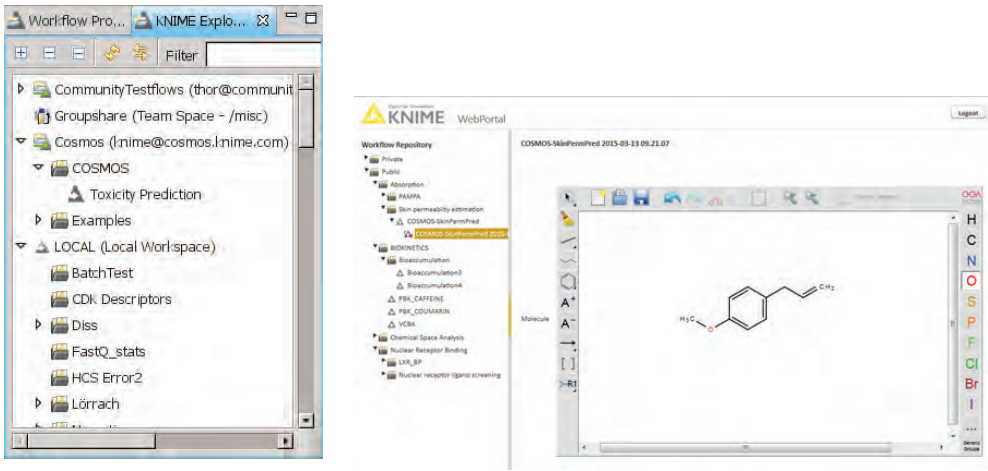

Figure 4.34 *Left: The KNIME Explorer offering a view on several storage locations for KNIME workflows on a user's desktop. Right: The COSMOS KNIME WebPortal to execute workflows through a web browser.*

The workflows input and output for the WebPortal can be parameterised using "Quickforms". In addition, report templates created with the KNIME Report Designer that are uploaded together with their associated workflow to the shared repository on the KNIME Server can be filled dynamically with data and downloaded in various formats.

The user group management and permission system has been extended to allow a closed user group access to dedicated private folders on the server to share the workflows still under development. The finalised models can be moved to a public folder, which is accessible for freely registered users.

Versioning and archival functionalities were integrated in the KNIME Server updates. Starting with KNIME Server 3.8, a history of overwritten and deleted workflows is kept to make restoring of previous workflows possible. So-called snapshots save the complete state (i.e. data and metadata) of a workflow or file at this moment in time, they can be downloaded locally or replace the current version of a workflow/file. An additional new feature of the server is a recycle bin, which allows downloading or restoring of deleted items (workflows, files, and workflow groups) from the bin into any place on the server.

New features to implement models into workflows included for example the integration of R, allowing interactive modelling of R scripts, as well as Python to be used for the KNIME nodes. Furthermore, new database nodes were made available, and a new Table Validator node checking the input table to correspond to an expected structure.

The COSMOS KNIME WebPortal. The COSMOS KNIME WebPortal allows access to the KNIME Server and execution of workflows through a web interface from any recent web browser without installation of the software locally and without knowledge of the KNIME workflows as such. Thus, as opposed to access from the KNIME desktop, end-users that only want to run a workflow with custom data can access the server via the KNIME WebPortal. The web interface lists all publicly accessible **COSMOS** workflows.

The WebPortal allows for a step-by-step execution. Each step asks for user input, such as files or model parameters, potentially also dependent on previous inputs. After all inputs have been provided, the workflow is executed and the results can be downloaded as files and/or graphical reports are generated as summaries.

The COSMOS KNIME WebPortal is freely accessible at the URL http://knimewebportal.cosmostox.eu, and is supported by COSMOS Space (http://cosmosspace.cosmostox.eu), a facility to share predictive toxicology resources. COSMOS Space provides free registration to login to the COSMOS KNIME WebPortal (log-in credentials are the same as for COSMOS Space), hosts the workflow documentation as well as user guidance and will link, in the future, to web tutorials which will be made available for the different workflows (see *Figure 4.35*).

Figure 4.35 *The COSMOS KNIME WebPortal allows for the easy execution of the KNIME models in a web browser without installation of any software. It is supported by COSMOS Space which includes the workflow documentation and user guidance.*

COSMOS models implemented into KNIME. A series of *in silico* models has been developed and extended within the **COSMOS** project to support safety assessment, with focus on cosmetics-related substances, for which the dermal exposure route is important.

Models developed within **COSMOS** comprise

- biokinetics models;
- models for absorption;
- structural alerts and models for toxicity;
- tools for cosmetics/chemical space analysis.

Physiologically-based kinetic (PBK) models simulate relevant time profile concentrations during absorption, distribution, metabolism and excretion within the body. Coupled with *in vitro* dynamics to *In Vitro* to *In Vivo* Extrapolation (IVIVE) models, they relate an external exposure dose to intracellular concentrations and target-organ levels.

The major route of exposure for cosmetics is following dermal application, while the majority of available toxicity data are obtained from oral administration. Thus, models for skin permeability and gastrointestinal absorption contribute to the extrapolation from oral to dermal exposure.

Computational toxicity prediction models are developed based on chemical categories, read-across and structure-activity relationships ((Q)SAR), anchored in an Adverse Outcome Pathway (AOP) framework, which links chemistry to toxicological effects.

Chemical space analysis of datasets is a preliminary step before modelling. It includes Principal Component Analysis (PCA) and distribution analysis, based on various descriptors, as well as functional groups profiling.

In particular the **COSMOS** models developed include

- Models for biokinetics: over 20 workflows for
  - PBK models for oral, dermal, inhalation exposure to human and rat
  - *in vitro* to *in vivo* extrapolation (IVIVE)
  - virtual cell-based assay (VCB)
  - human bioaccumulation factor models, general and specific for chemicals
- Models for absorption:
  - skin permeability QSAR
  - oral (PAMPA) absorption QSAR

- Structural alerts and models for toxicity:
  - nuclear receptor binding potential prediction:
    - Partial Least Squares Discriminant Analysis (PLS-DA) classification models for LXR binding
    - prediction of potential binding to nuclear receptors (including LXR, PPAR) related to steatosis.
  - structural alerts profilers:
    - 108 alerts for covalent binding to proteins (Molecular Initiating Event for endpoints such as hepatotoxicity)
    - 85 for DNA binding
    - 32 for phospholipidosis and 16 for other liver toxicity outcomes
    - 30 for mitochondrial toxicity.
- Cosmetics/chemical space analysis tools
    - including Principal Component Analysis (PCA) and distribution analysis (based on various descriptors), as well as functional groups profiling.

The models have been and are being implemented into KNIME workflows. A number of the workflows have already been transferred into user-friendly WebPortal versions, allowing non-experienced users to execute workflows in a web-browser with pre-selected configuration options, without installation of local software. The results are available as downloadable files (e.g. Excel sheets) and pdf reports.

However, the desktop versions of the KNIME workflows will also be made accessible via the KNIME Server, for users using the installed KNIME software. Thus, proficient users will have the possibility to adapt the workflows to their needs, if applicable.

Example of a KNIME workflow: PBK model for caffeine and coumarin. A physiologically-based kinetic model is a mathematical model for predicting the absorption, distribution, metabolism and excretion (ADME) of a compound. It can be used to simulate relevant time profile concentrations of selected chemicals and their metabolites, in view of supporting *in vitro* testing design as well as chemicals safety assessment.

In this work, the main goal was to develop and optimise computer-assisted physiologically-based toxicokinetic (PBK) and dynamic (PBD) models to predict the biological and toxicological effects of chemicals in humans under single and repeated exposure conditions,

by applying the modelling approach to selected chemicals found in cosmetics products such as caffeine and coumarin (*Gajewska et al., 2014; 2015*). A schematic representation of the general PBK model is given in *Figure 4.36*. The PBK model attempts to mimic the human body compartments including the blood circulation to and from the organs and the movement of a chemical substance throughout the compartments (*Gajewska et al., 2014; 2015*).

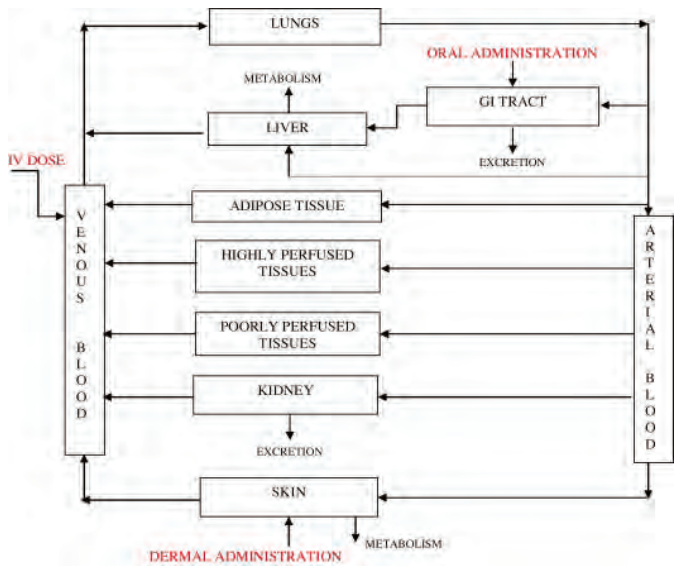

Figure 4.36 *General structure of the PBK mode (source: Gajewska et al., 2014).*

Exposure routes used in the present PBK model are: oral, dermal and inhalation. In the human PBK model for oral absorption, the human gastrointestinal (GI) tract is represented by six sub-compartments, dissolution from matrix, stomach emptying rate and a first order rate of absorption from stomach, small and large intestine are taken into account. Four compartments were used for dermal exposure, i.e. surface compartment for the product formulation, *stratum corneum*, viable epidermis and dermis perfused by blood, plus hair follicles for caffeine. The respiratory tract consists of three main compartments. The model, additionally, takes into account the blood/air partition coefficient, Ostwald solubility in lungs and the rate of dissolved gas removed from lungs due to blood perfusion on overall model prediction performance.

In order to obtain the best simulation of ADME processes in humans, three structurally different PBK models were developed (PBTK1, 2, 3). They are represented as a set of non-linear differential equations with clearance rates (either liver or kidney) being dependent on a chemical concentration within a given organ/tissue and its tissue-to-blood partition. The schematic representation of the main organs considered applies to all three models and the differences between them are in the GI tract and skin compartments.

For the human oral model, the most sensitive parameters (GIT dissolution rates, first order uptake rate constants and metabolism parameters for liver) were optimised using measured human data. Liver metabolism parameters from the literature were optimised for *in vivo* blood concentrations using human data for the main metabolites. Physiological parameters independent of the chemical and part of the model equations as constants were taken from *Brown et al.* (*1997*).

The mathematical equations were programmed in the R language and the models subsequently implemented into KNIME workflows (see *Figure 4.37*). The models to simulate relevant time profiles concentration of caffeine, coumarin and their metabolites for human, rat and *in vitro*, for different exposure (oral, dermal) and target organs (blood, liver, lungs), are freely available in the COSMOS KNIME WebPortal. The user can choose the model to run and can set the variables time, dose, volume. The output of the model calculations are concentration time profiles of the substance and the metabolites (*Figure 4.38*) and a table with the area under the curve (AUC) and C_{max} values in blood, liver and lungs.

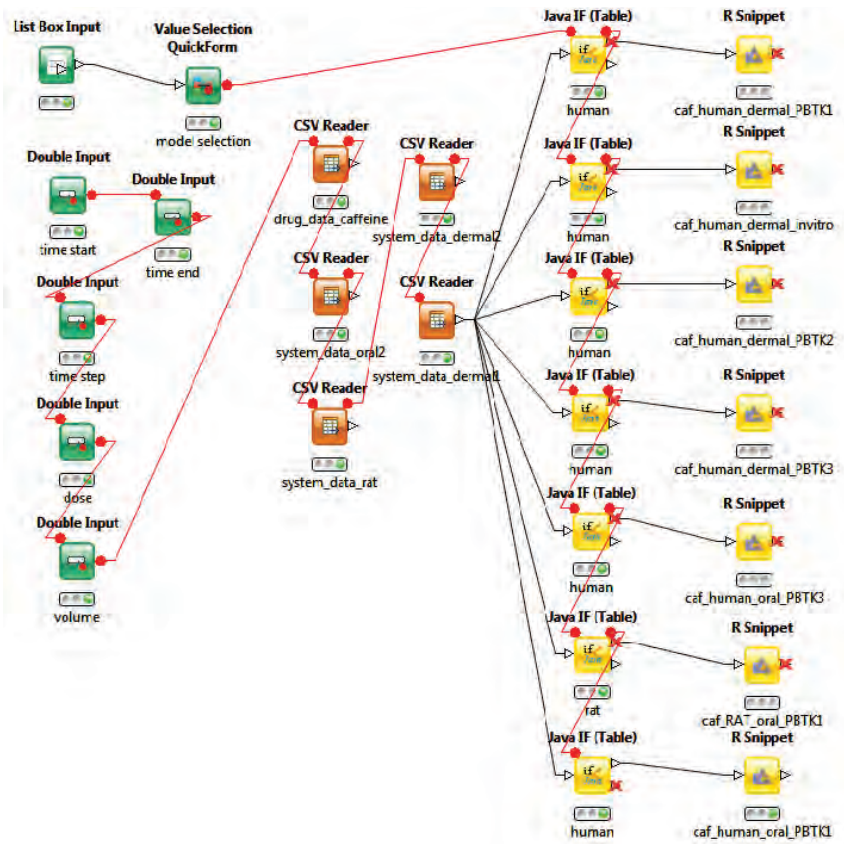

Figure 4.37 *KNIME workflow of the PBK model for caffeine.*

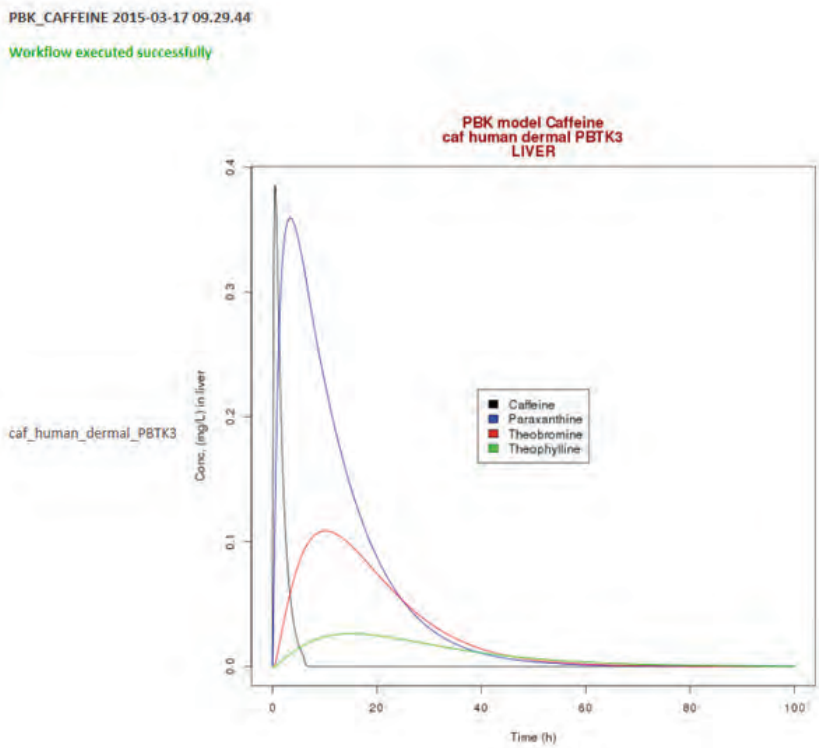

Figure 4.38 *Result of running the human dermal PBK KNIME workflow for caffeine in the WebPortal: concentration time profiles of caffeine and its metabolites in the liver.*

4.5.4 Innovation

The flexible KNIME software is well suited as an adaptable and transparent platform to make the models developed in the **COSMOS** project publicly and freely available. With the WebPortal versions the models are easily accessible and executable through a web browser application even for a non-proficient user of the software. At the same time, the KNIME desktop versions are offering the more expert user the possibility to adapt the workflows to their needs, where applicable, and execute them locally on their computer, which allows to securely use confidential data and chemical structures.

The openness and flexibility is a major advantage, in line with the aim of **COSMOS** to provide open and transparent models, in the spirit of the OECD Principles for Validation of QSARs. Furthermore, through linkage with the documentation in COSMOS Space, they are also well documented and background information and user guidance is provided. The COSMOS KNIME models constitute thus a true legacy of **COSMOS**, which will be usable beyond the end of the project and provide tools to support the chemical safety and risk assessment.

4.5.5 Contributions to the SEURAT-1 Case Studies

COSMOS work on computational predictive models feeds into the case studies, as well as the COSMOS Database which is providing information for chemical selection and toxicity data.

COSMOS is leading the level 2 case study on developing chemotypes for mitochondrial toxicity (see section 3.4.5). The aim of this case study was to develop an *in silico* profiler consisting of a series of chemotypes to identify compounds with the ability to induce mitochondrial toxicity. The objective was to implement the chemotypes developed into KNIME nodes or the ChemoTyper software to be used for grouping chemicals into categories within the AOP paradigm, allowing for the prediction of organ-level toxicity via read-across.

COSMOS has also contributed to the level 2 case study on the use of biomarkers to substantiate read-across prediction, investigating whether biomarkers from '-omics' investigations can increase the mechanistic knowledge on the individual compounds. **COSMOS** was involved in contributing to the 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. Similarly, **COSMOS** is contributing to the level 2 case study on Mode of Action based classification models for repeated dose liver toxicity, 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**.

Another level 2 case study led by **COSMOS** is investigating the use of molecular modelling approaches to predict potential binding to nuclear receptors involved in the development of liver steatosis, with the example of the Liver X receptor (LXR) and the Peroxisome Proliferator-Activated Receptor γ (PPARγ; see section 3.4.6). This case study is a proof-of-concept that molecular modelling methodologies, usually used in drug discovery, can be employed in predictive toxicology as part of an integrated strategy.

COSMOS is also intensively involved in the cross-cluster level 3 read-across case study (see section 3.5.1), firstly by contributing to the formulation of the strategy for structuring and reporting read-across predictions of toxicity (*Schultz et al, 2015*), and in on-going work by contributing to analyse and summarise the case study scenarios. Furthermore, COSMOS DB and other resources including ToxCast were mined for available repeated dose toxicity data, as well as the new *in silico* profilers developed in the project being applied for similarity analysis and to support the categories of analogues.

COSMOS has also contributed to the cross-**SEURAT-1** level 3 *ab initio* case study on integrating the level 2 case study results for a quantitative mechanistic safety assessment, by supporting the selection of the cosmetics-related case study substance and by screening the case study chemicals with computational methods (see section 3.5.2). Moreover, with

exposure considerations and the internal concentration in *in vitro* measurements being crucial aspects of the case study, **COSMOS** has provided PBK modelling for the case study substances methotrexate, valproic acid and piperonyl butoxide, and will further support the link to risk assessment when experimental *in vitro* toxicity data will become available from the other **SEURAT-1** projects.

The **COSMOS**-led level 3 case study on Thresholds of Toxicological Concern (see section 3.5.3) is evaluating the extension of the TTC approach to cosmetics-related chemicals in view of its use for safety assessment for cosmetics. This is achieved by creating a new non-cancer TTC dataset of NOAEL values, enriched with appropriate data and therefore more suitable for the evaluation of cosmetics ingredients, as well as by addressing the difference in exposure scenarios and bioavailability issues, such as dermal absorption and skin metabolism, for the oral-to-dermal extrapolation of the TTC.

4.5.6 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'. The overarching cross-cutting activities regarding AOPs are indeed a major theme within **SEURAT-1** with **COSMOS** efforts on mitochondrial toxicity being highlighted.

COSMOS continues to support **SEURAT-1** case studies on read-across through the searching of COSMOS DB, as well as other sources, for data, the provision of chemoinformatics support for similarity analysis, and applying the new *in silico* profilers developed in the project. Furthermore, **COSMOS** was involved in formulating the strategy for structuring and reporting read-across predictions of toxicity (*Schultz et al, 2015*) and is contributing to analyse and summarise the case study scenarios. Similarly, **COSMOS** has contributed to the case study on *ab initio* safety assessment by supporting the chemical selection and profiling the case study compounds with computational methods.

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

Threshold of Toxicological Concern (TTC) approach to cosmetics-related chemicals and *in silico* tools for *in vitro* to *in vivo* extrapolation (IVIVE).

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. Together with COSMOS DB, in which it is embedded and which has been made publicly available, it has been used as reference and for chemical selection for **SEURAT-1** investigations. Throughout the fourth year **COSMOS** interacted with ToxBank to enabling the interoperability between COSMOS DB and the ToxBank data warehouse.

The **COSMOS** work on physiologically-based kinetic (PBK) models and *in vitro* to *in vivo* extrapolation led to the development of a case study centred around 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.

The cell-based assay model, implemented using an open source platform (KNIME/R) has been completed and made available to interested partners in 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.

COSMOS also contributed to the first read-across workshop on 29-30 April 2015 in Ispra, Italy, discussing the read-across case study scenarios with the support of **SEURAT-1** external experts, as well as to the *ab initio* case study workshop on 9-10 October 2014 in Ispra, evaluating how **SEURAT-1** methods can contribute to chemical risk assessment for human repeated dose toxicity. **COSMOS** partners also participated in the '**SEURAT-1** meets MIP-DILI' preparatory teleconference on 16 December 2014 (see section 5.3).

4.5.7 Expected Progress within the Fifth 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 (http://cosmosdb.cosmostox.eu)

has been successfully 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 fifth year of the project and updates will be released with the inclusion of further data on e.g. skin permeability. This will lead to a comprehensive open database by the end of the project. Furthermore COSMOS Space (http://cosmosspace.cosmostox.eu), which facilitates user interaction and sharing of predictive toxicology resources, was launched officially in March 2015 with the documentation for the published COSMOS KNIME workflows and will further be populated and promoted.

COSMOS has compiled a dataset for Threshold of Toxicological Concern (TTC) analysis and this will make the data transparent for any possible further work. Specifically, in the fifth year, **COSMOS** will deliver the quality-controlled COSMOS TTC dataset of repeat dose NO(A)EL/LO(A)EL values. 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.

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 is being pursued further in the fifth year. With the markup language CSRML, it is possible to extend and expand the chemotypes and structural alert concept by inclusion of other relevant physicochemical properties. The dataset containing physicochemical 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 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 fifth 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. The first biokinetics and predictive toxicity models developed in **COSMOS** have been made publicly accessible and executable through the COSMOS KNIME WebPortal (http://knimewebportal.cosmostox.eu), with the official launch at SOT 2015. Documentation of the models is provided through COSMOS Space. The KNIME Server is

being updated further in the fifth year of the project and will allow more bespoke customisation of the WebPortal. Additional functionality and usage improvements will be added.

During the fifth year **COSMOS** is continuing to actively contribute to the **SEURAT-1** cross-cluster Case Studies.

4.5.8 Future Perspectives

Computational modelling is at the heart of the modern toxicological paradigm. The **COSMOS** project within the **SEURAT-1** Research Initiative is providing 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** is providing 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 Ver 1.0 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 is developing **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. Kinetics modelling (e.g. through physiologically-based (PBK) 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 will all be amongst the strategic requirements for future research in 21st Century toxicology.

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. There is a strategic requirement to develop and utilise open and transparent platforms, such as KNIME, to capture and implement modelling processes in future research. 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

Bucher, J., Diaz Ochoa, J.G., Mauch, K., Terfloth, L. (2015): Coupled modelling of PBPK and toxic mechanisms of action of valproic acid in liver. Poster award at the SEURAT-1 5th Annual Meeting 2015, 21–22 January 2015, Barcelona, Spain.

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, Spain.

Jereva, D., Al Sharif, M., Diukendjieva, A., Alov, P., Pencheva, T., Tsakovska, I., Pajeva, I. (2014): Nuclear ERα and PPARγ: receptor- and ligand-based analysis. Poster award at the 16th Congress of the European Neuroendocrine Association, 10-13 September 2014, Sofia, Bulgaria.

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, Portugal.

Nelms, M.D., Enoch, S.J., Fioravanzo, E., Madden, J.C., Meinl, T., Richarz, A.-N., Schwab, C.H., Worth, A.P., Yang, C., Cronin, M.T.D. (2012): Strategies to Form Chemical Categories from Adverse Outcome Pathways. Poster award at the SEURAT-1 2nd Annual Meeting 2013, 8–9 February 2012, Lisbon.

Paini, A., Benito, J.V.S., Gajewska, M., Worth, A.P., Zaldivar Comenges, J.M. (2013): Human Bioaccumulation Potential Simulated in R and Implemented in KNIME. Poster award at the SEURAT-1 3rd Annual Meeting 2013, 6–7 March 2013, Lisbon, Portugal.

Richarz, A.-N., Neagu, D., Yang, C., Fioravanzo, E., Péry, A.R.R., Berthold, M.R., Cronin, M.T.D. (2012): COSMOS: An International Cooperative Project Developing Computational Models for Repeated Dose Toxicity. Poster Award 2012 at the European Partnership for Alternative Approaches to Animal Testing (EPAA) Annual Conference "Global Cooperation on alternatives (3Rs) to animal testing", 16 November 2012, Brussels, Belgium.

Richarz, A.-N., Enoch, S.J., Hewitt, M., Madden, J.C., Nelms, M.D., Przybylak, K.R., Yang, C., Berthold, M.R., Meinl, T., Ohl, P., Cronin, M.T.D. (2013): Flexible computational workflows to predict toxicity. Poster Award at the UK-QSAR and ChemoInformatics Group Autumn Meeting, AstraZeneca, 15 October 2013, Alderley Park, England.

Teng, S., Barcellini, S., Beaudouin, R., Rahmani, R., Péry, A. (2014): TK/TD modelling to analyse real time hepatotoxicity data for cosmetics. Poster award at the SEURAT-1 4th Annual Meeting 2014, 5–6 February 2014, Barcelona, Spain.

Teng, S., Péry, A., Kovarich, S., Bois, F. (2015): SEURAT-1 Level 3 *ab initio* case study: PBPK predictions of methotrexate and valproic acid tissue concentration in humans. Poster award at the SEURAT-1 5th Annual Meeting 2015, 21–22 January 2015, Barcelona, Spain.

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

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4.6.1 Introduction and Objectives

Validated alternative assessment methods for long-term systemic toxicity are urgently required to cope with the complete ban (enforced 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** develops and establishes 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 are identified including molecular initiating events (MIE). The models are multi-scale, from molecular to cellular and tissue levels.

Since testing on the target organisms (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 & Andersson, 2008a; 2008b*). For validation purposes, and for the development of new techniques, we also use 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 are monitored by determining '-omics' data (epigenomics, transcriptomics, proteomics, metabolomics, fluxomics) at various time points. The design of experiments incorporates 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 are implemented. Together with curated literature and genomic data, these toxicological data are 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 are characterised by 3D cryo-electron tomography and light/confocal microscopy. We 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 are 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 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 are 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 illustrate how computer models calibrated with *in vitro* experiments could be used in combination with human parameters to predict the possible toxicity in humans.

4.6.2 Main Achievements and Challenges in the Fourth Year

NOTOX AOP Case Study Valproic Acid

NOTOX is very much focusing on AOP-oriented in-depth case studies (see also section 4.6.4). In 2014, multi-omics data of a joint experiment applying valproic acid (VPA) on 2D HepaRG cultures were generated. The measurements include liver specific data, expression analysis, proteomics (1300 proteins quantified), epigenetics and metabolomics. Clear trends were observed in viability, transcriptomic, proteomic, metabolomic and epigenetic analyses. An overarching bioinformatics analysis of all data is still on-going. Based on a literature

study and own analyses, a first kinetic model of the action of VPA was generated by INSIL generating an expected response. This model comprises VPA and metabolites thereof, and a series of important proteins triggering the adverse outcome. Important proteins are SREBP1a stimulating fatty acid synthesis via ACACA and FASN and PPARa whose synthesis is reduced thus eventually reducing fatty acid metabolism. The model also includes inhibitory and toxic effects of VPA metabolites. However, comparison of the model behaviour and experimental data still show some disagreement. A potential reason is in different time scales of the primary toxic effect and later compensatory response of the cells. Correspondingly, the model is further developed by checking literature data, particularly applying ToxWiz of CCNet, and by the introduction of additional relationships based on our own data. We expect more insight in the mechanism by the thorough overarching analysis of now existing multi-omics data mentioned above.

Parallel experiments using 3D spheroids of HepaRG cells to study effects of VPA over a period of 2 weeks were conducted and it was found that cell types remain constant irrespective of drug application but EC_{50} decreased with time confirming earlier experiments. Glutathione decrease indicated oxidative stress. Carnitin palmitoyl transferase (CPT1A) expression was increased by VPA. Methods for proteome analysis for 3D spheroids were developed (see below) and are presently applied to samples of the 3D spheroids. Further details are reported in section 4.6.3.

Proteomic Characterisation of HepaRG Cells

Batch to batch reproducibility: The HepaRG cell line was further characterised with a strong focus on the proteome using the complementarity of two different proteomics strategies, namely GeLC-MS/MS and 2D-gel electrophoresis combined with MS. As expected, not all proteins identified from the 2D-gel (approx. 850) were identified in the GeLC-MS/MS experiment (approx. 1500) and *vice versa*, underlining the complementarity of the two approaches. Based on approximately 2000 identified proteins in total, HepaRG cells exhibit a phenotype very similar to that of functional liver cells. To verify the reproducibility of the production/differentiation process of the cell line, three different HepaRG-batches were compared using 2D-DIGE and by label-free quantitative LC-MS/MS by means of spectral counting. Two differentially expressed proteins were found in the DIGE experiment and 25 proteins seen by LC-MS/MS exhibited altered abundance, particularly in one of the three batches. However, fold changes were always less than two-fold, indicating good batch-to-batch reproducibility of our selected *in vitro* model system.

The extracellular proteome ('Secretome') of HepaRG: Proteins secreted by hepatocytes into the extracellular space – i.e. the secretome – are a valuable source for biomarker candidates of DILI, especially since they are readily accessible in (pre)clinical *in vitro* and *in vivo*

studies by analysing culture supernatants or blood samples, respectively. In order to study this important HepaRG sub-proteome, we established and optimised a shotgun-proteomic workflow. We successfully incorporated an immunodepletion step during sample preparation in order to remove the abundantly secreted protein albumin, and thus increased the number of identified secreted proteins by more than 100%. Using nanoLC-MS/MS after liquid digestion of an albumin-depleted test sample (conditioned culture medium of HepaRG cells), more than 200 proteins were identified. 60% of these proteins were known to be located in the extracellular space according to the Gene Ontology (GO) database, thus supporting the fact that we identified mostly proteins belonging to the secretome of HepaRG cells, with a very low level of contamination from intracellular proteins. The reproducibility of sample preparation including immunodepletion and subsequent in-solution digest was verified.

Organotypic 3D spheroid cultures: The applicability of the elaborated quantitative proteomics workflows to 3D-spheroid cultures of HepaRG was verified. Since they consist of 2000 cells only, sample material available from this type of cell culture is very limited and proteomics analyses impossible without pooling of several spheroids. The number of spheroids necessary for performing proper protein extraction as well as digestion and subsequent mass spectrometry-based analyses was determined. Furthermore, the sample preparation was optimised regarding spheroid harvest before sample transport and protein extraction. It is currently not possible to omit foetal bovine serum during generation of spheroids using inSphero's 'hanging-drop' method and hence, it was mandatory to ensure optimal removal of serum and the bovine proteins contained therein. The developed methods are currently applied to samples from an experiment where HepaRG spheroids were treated with VPA as a complement to the consortium-wide case study on steatosis, which was performed using 2D-monolayer cultures of HepaRG.

3D Spheroids – Functional Imaging and Quantification of Hepatocyte Morphology

Techniques for the in-depth characterisation of liver tissue und hepatic spheroids using confocal microscopy were further developed and applied to monitor the transport of dyes into spheroids. Bile canalicular structures could be visualised (*Figure 4.39*), which serves as an important input for agent-based modelling of structure and function of 3D spheroids.

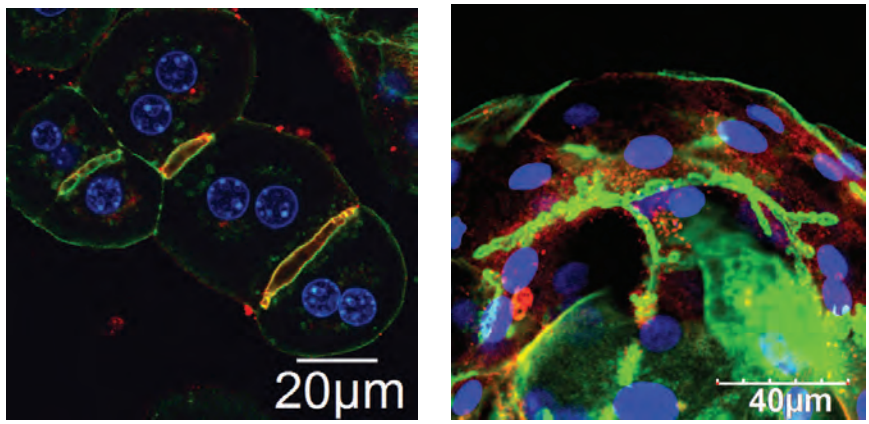


Figure 4.39 *Immunostaining for bile canaliculi in sandwich culture (cells embedded in a collagen matrix) and spheroids: Green fluorescence shows the bile canalicular structures visualised with an antibody against the marker protein DPPIV. Both cultivation systems, left sandwich culture - right spheroid, display cell polarity with bile canaliculi at the cell interface. Nuclei in blue and plasma membrane in red.*

Toxicological testing compounds of interest have to penetrate the plasma membrane to reach the cytosol where usually toxic effects are manifested or where compounds are converted into toxic metabolites. So far the efficiency of compound uptake in different cultivation systems has not been estimated. Recent development in fluorescent probes enables to study their distribution in living cells using confocal microscopy. Using this technique a set-up to evaluate compound uptake capabilities of sandwich cultures and spheroids was established using a two-photon microscope. In order to be able to visualise cell morphology, primary hepatocytes expressing ubiquitously the red fluorescent protein tomato were taken, which comprises a lipid anchor and is therefore targeted to cellular membranes. As a passively diffusing model compound Hoechst 33258 was chosen, which gains blue fluorescence upon intercalation into DNA. As a second model compound CMFDA was chosen. CMFDA is also passively taken up, but has to be metabolically activated by esterases located in the cytosol to become a fluorophor. The metabolites of CMFDA are then eliminated via the bile canalicular system.

First, the uptake of Hoechst 33258 by hepatocytes in sandwich culture was studied (*Figure 4.40*). Imaging started with a medium change containing 0.05 mM Hoechst.

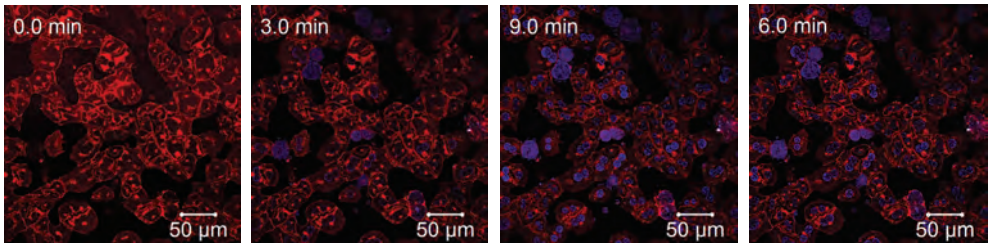


Figure 4.40 *Passive diffusion of Hoechst in sandwich cultures: Primary mouse hepatocytes (td-tomato) were cultured for 4 d in collagen sandwiches and exposed to 0.05 mM Hoechst (nuclear marker). Compound uptake was measured in a time dependent manner using two-photon microscopy.*

Similarly, hepatocytes cultivated in spheroids for 5 days (*Figure 4.41*) using Gravity^{plus} plates were placed on collagen coated culture plates for fixation, and afterwards exposed to 0.05 mM Hoechst.

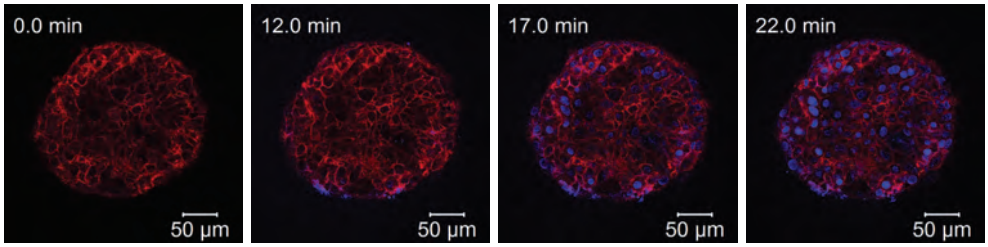


Figure 4.41 *Passive diffusion of Hoechst into spheroids: Spheroids of primary mouse hepatocytes (td-tomato) were fixed after four days in cultivation on a collagen layer and were exposed to 0.05 mM Hoechst (nuclear marker). Compound uptake was measured in a time dependent manner using two-photon microscopy.*

In spheroids the outer layer of hepatocytes was stained with Hoechst after around 5 min, but the penetration throughout the spheroid is limited and took more than 20 min to reach into the inner core. In contrast the intercalation by hepatocytes in sandwich culture was homogeneous and fast, nuclei became fluorescent in 2 min and were saturated after 5 min. Comparing these results with *in vivo* experiments conducted under the umbrella of another project it becomes evident that the uptake *in vivo* is even faster (data not shown).

Analogous experiments were carried out with the dye CMFDA (5-chloromethylfluorescein diacetate; 3.2 μM) in sandwich cultures (*Figure 4.42*) and in spheroids (*Figure 4.43*). Both cultivation systems were able to convert CMFDA into the corresponding acid that exhibits green

fluorescence and eliminate this compound as expected into the bile canalicular structures. Similar to the Hoechst exposures, the uptake in sandwich cultures was fast – activation in the cells occurred within 3 min and after 8 min it was visible in the bile canaliculi. In spheroids the outer cell layer showed very fast CMFDA metabolism (within 1 min), whereas cells in the inner part of the spheroid were CMFDA positive from 10 min onwards. Bile canalicular structures in the spheroid were transient and visible all over the spheroid from 10 min after CMFDA administration on, peaking between 20-30 min.

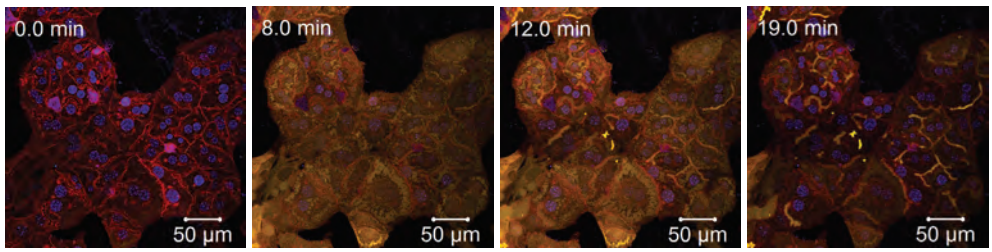


Figure 4.42 *Uptake of CMFDA in sandwich culture: Primary mouse hepatocytes (td-tomato) were cultured for 4 d in collagen sandwiches and exposed to 3.2 µM CMFDA (metabolite in green). Compound activation and elimination was measured in a time dependent manner using two-photon microscopy.*

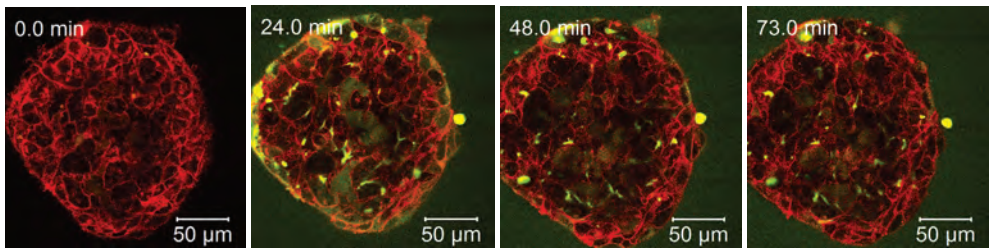


Figure 4.43 *Uptake of CMFDA into spheroids: Spheroids of primary mouse hepatocytes (td-tomato) were fixed after four days in cultivation on a collagen layer and were exposed to 3.2 µM CMFDA (metabolite in green). Compound activation and elimination was measured in a time dependent manner using two-photon microscopy.*

In conclusion, both *in vitro* systems, hepatocyte sandwich cultures and spheroids can be used to study bile canalicular transport. CMFDA is taken up by hepatocytes, converted into the corresponding acid that exhibits green fluorescence and is transported to bile channels via the MRP2 transporter. This has a great potential for monitoring of cholestatic compounds. However, it should be considered that transport processes are much faster in livers *in vivo*.

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

NOTOX involves new methods of electron microscopy, Cryo-Electron Tomography (Cryo-ET) and Focused Ion Beam/Scanning Electron Microscopy (FIB/SEM). We developed a prototype of a data analysis workflow in combination with visualisation algorithms in order to demonstrate core capabilities in identification of macromolecular structures and organelles. Furthermore, for a second class of image data obtained by FIB/SEM tomography on liver cell spheroids, a segmentation protocol was explored to be able to identify individual cellular sub-structures, like cell nuclei, cell membrane as well as bile structures forming in the inter-cellular space. The 3D image data on various levels of resolution allows to study the binding of molecules to large-scale entities like ribosomes on the one side and on the other will allow to elucidate the 3D-fine structure of spheroids that are successfully used for toxicity assessment. These improvements of methods for Cryo-ET were achieved both in the experimental procedures as well as in computation (*Dahmen et al., 2014; Voortman et al., 2014*). The specialised software development for the tomographic reconstructions of electron tomography data, performed during the **NOTOX** project, was generalised, consolidated and put in the form of a publishable, long-term usable software package called 'ettention'. The ettention software package will be released in form of an OpenSource package.

Fusidic acid, a steroid type bacteriostatic antibiotic, was applied to study its interaction with the EF-G-GDP-ribosome complex. The fusidic acid inhibits the coupled movement of mRNA and tRNAs through the ribosome by blocking elongation factor EF-G-GTP dependent translocation step, thus preventing consecutive codons reading (*Gao et al., 2009; Pulk and Cate, 2013; Stark et al., 2002*).

The possibility to sort out drug-induced conformational states of macromolecular complexes from a mixed population is one of the recently introduced features of the single particle analysis (SPA) technique. We demonstrate that it is possible to deliver sub-20A resolution on a fully asymmetric complex as well as to reliably distinguish drug-induced conformational changes from a mixed population. Additionally, we propose an improved CTF correction scheme for tilt-series based on key acquisition-time events. The 70S subunit of *E. coli* ribosome was treated with a below-saturation amount of fusidic acid and hence induced the partial association of EF-G to the ribosome. We obtained a mixture of 70S alone and 70S locked to EF-G (*Figure 4.44*). Counting ribosomes in different states, we found that around 85% of the 70S (~2400 particles) were bound to the EF-G stalled in the late elongation state by the addition of fusidic acid whereas the rest of the 70S (~350 particles) was not bound to any factor.

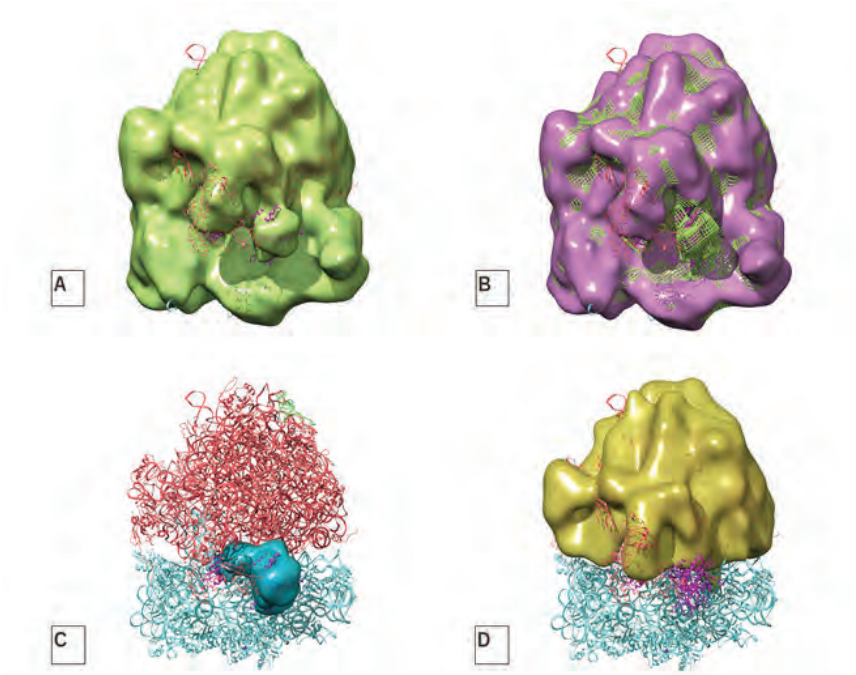


Figure 4.44 *cryo-ET Structures of ribosomes. Structures of E. coli ribosomes with docked inside the X-ray structures of Thermus thermophilis 70S. In green is shown the EM-map of the 70S+EF-G (surface panel A and green mesh panel B), in magenta the density relative to the 70S without EF (surface panel B), where we have colored in yellow (panel D) the density of the 50S only. The small density in turquoise (panel C) corresponds to the EF-G only. The X-ray structures docked in the EM map shows ribbons of the 50S in green, the 30S in cyan, and the EF-G in pink. A) 70S+EF-G. Most of the density of the cryo-ET map, including the one relative to the EF-G, is occupied by the pdb model, as expected. B) Overlap of the 70S+EF-G and 70S only. The density relative to the EF-G is present only in the map 70S+EF-G (mesh). C) Subtraction, made with Chimera in between the 70S+EF-G (green map) and 70Sonly (magenta map). D) Map of the 50S only.*

Towards Multi-Scale Liver Modelling *in vivo*: Model Components and Simulation Tools

Multi-scale modelling aims at a realistic *in vitro* to *in vivo* extrapolation. Tissue architecture (monolayer or spheroid *in vitro* versus highly organised liver lobule *in vivo*) and molecular networks (e.g. different expression levels of drug-metabolising enzymes *in vitro* and in *in vivo*) are key elements. The *in silico* framework we are developing in **NOTOX** permits to vary the mechanisms considered and hence test different models against each other and against data,

and to identify the correct model following a systematic strategy involving iterations between experiments and modelling (*Drasdo et al., 2014*). As each iteration is time-consuming, the creation of efficient tools is an important component in modelling-guided toxicology assessment (D'Alessandro et al., 2014).

Model components. The key components of a model are the chemical reactions and transport processes affecting the concentration of molecular compounds in the environment and within the cells (*Figure 4.45A*), and the spatial arrangement of the cells *in vitro* and *in vivo*, and their dynamics. To enable the representation of tissue architecture, our models are spatial and three-dimensional. Each individual cell is modelled in order to represent differences between individual cells, which is necessary to generate the variability of cells upon response to chemicals (*Figure 4.45B*). Movement of cells is mimicked by an equation of motion, one for each cell. This permits to calculate the position of a cell as a consequence of all forces acting on it and its own micro-motility. Cells in the simulation can grow, divide, and die. At the cellular level, growth and proliferation, death, the strength of adhesion or cohesion, as well as other parameters can be predicted dependent on the concentrations of the chemical of interest.

Local averaged concentrations determine chemical reactions occurring in the cell and in the extracellular space. In the extra-cellular space, molecules can be transported by diffusion or with flow. *In vivo*, the smallest anatomical and functional repetitive units are the liver lobules. Blood enters by the portal veins of a lobule, flows along the hepatocytes through the hepatic sinusoids and drains into the central vein of a lobule from where it is carried away from the liver. Our model represents the individual sinusoids and permits modelling of flow inside the sinusoids. So far, we assume Poiseuille flow with a Faehraeus – Lindqvist effect for small capillaries within the sinusoids (*Figure 4.45C, D*). The model is embedded in a simulation tool named 'TiSim' that together with its sister module 'TiQuant' forms the software package 'CellSys'. 'TiQuant' (Tissue quantifier) is the image processing and analysis tool for tissues at histological scale (*Hammad et al., 2014*) supplying the initial spatial setting for simulation.

The model was applied to predict the detoxification of ammonia from blood. This was previously modelled by a system of ordinary differential equations in two spatial compartments (peri-central and peri-portal). The volumes of the two compartments were inferred from a spatial model describing the degree of tissue damage during regeneration after massive CCl_4-induced peri-central necrosis (*Schliess et al., 2014*). The novel model replaces the two compartments by individual cells organised in a lobule architecture and permits to evaluate the reactions in time and space in a multi-level (multi-scale) model. The same model will be used to predict the concentration-dependent liver damage caused by acetaminophen.

Simulation software: TiSim. *CellSys-TiSim* (Tissue Simulator) is a modular software tool for simulating multi-scale, multi-cellular individual-based models that can be applied on all three major 64bit computing platforms: GNU/Linux, Microsoft Windows 7/8, and OS X.

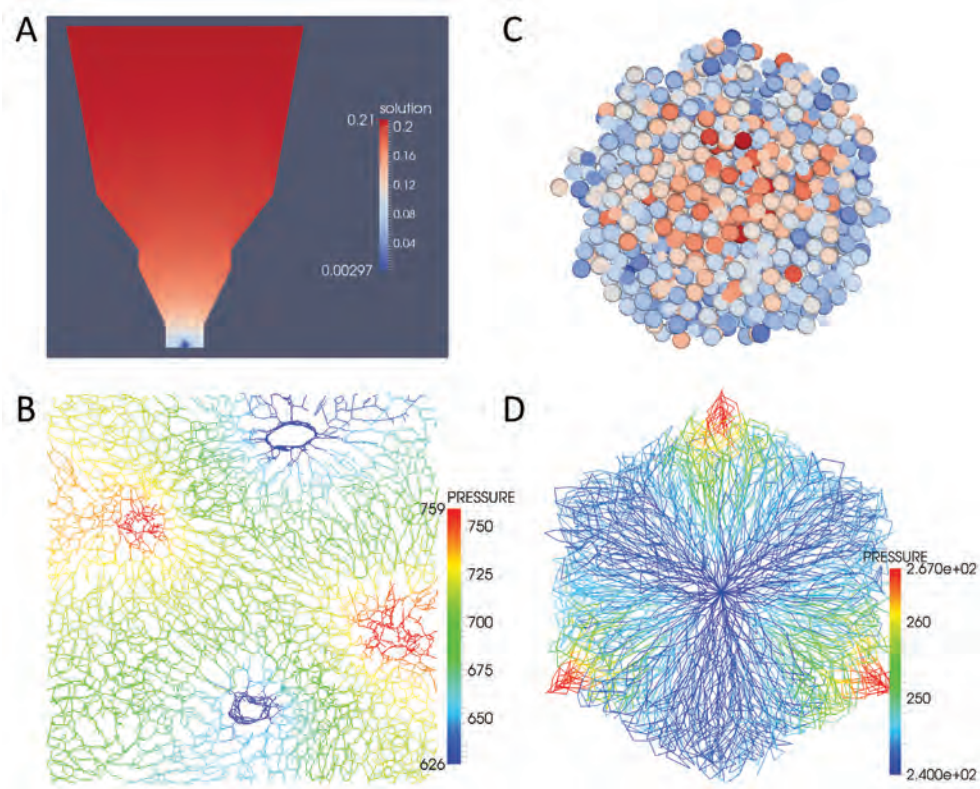


Figure 4.45 *(A) Oxygen profile in a 'trap'-well containing a multi-cellular spheroid at the bottom. Oxygen concentration is high at the top, where oxygen from the air dissolves into the medium, and low at the bottom were it is consumed by the spheroid. Scales units are mmol/L. (B) CYP enzyme levels (red=high, blue=low) for individual cells of spheroid subjected to an oxygen gradient. (C) Pressure distribution inside a real blood vessel network reconstructed from confocal micrographs, with highest pressures in the portal veins (in red, set as P=10Pa) and lowest pressures in the central veins (in blue, set as P=0Pa). (D) Pressure distribution inside a representative liver lobule, with highest pressures in the portal veins (in red, set as P=10Pa) and the lowest pressures in the central vein (in blue, set as 0Pa). A representative liver lobule is constructed by sampling from parameters used to quantify confocal micrographs (Hoehme et al., 2010).*

Metabolic and Signalling Pathway Model

A significant set of hypotheses for mechanisms of action was created and stored in a database with a visualisation tool that is accessible online to all partners. Furthermore, a comprehensive metabolic and signalling pathway model for the model compound acetaminophen was developed, which can be used as a knowledge base for creating hypotheses about toxic

effects and for further investigating which of these pathways might be involved in toxic effects following repeated exposures. The main use of this model within **NOTOX** is to interpret the output from transcriptomics, proteomics and metabolomics datasets, involving hundreds of modified molecular levels. The aim is also to identify pathways accounting for acetaminophen toxicity that are distinct from the traditional metabolic processes. In this way it supports quantitative modelling that is targeting toxicity extrapolation using simulation approaches. Generally, the methodology used here is applicable for other model compounds too, i.e. the model can be extended for additional compounds. The pilot project data were helpful to understand the experimental conditions and their impact on which pathways are likely to be affected. For other compounds to be tested in the **NOTOX** project, this model already has a software framework with its interactive functions that allow analysing several types of '–omics' data similar to the pilot project, and it contains information on many biological pathways that are linked to a xenobiotic response in the experimental system which can be readily used for other compounds in the **NOTOX** project and further enriched with information on pathways likely to be specific for the biological response to other compounds.

The computational model is available to consortium partners via an interactive browser for the ToxWiz database and in an SMBL (Systems Biology Markup Language) format that is compatible and can be used in alternative systems biology modelling tools.

4.6.3 Selected Highlight: The NOTOX Valproic Acid Case Study

Introduction

In **NOTOX**, we are developing models for the different adverse outcomes of drug-induced hepatotoxicity. These adverse outcomes include the steatotic liver. For studying the development of the steatotic liver we use valproic acid (VPA), a standard reference compound of the list prepared by ToxBank (Jennings et al., 2014). VPA is a very interesting compound to study since it is broadly used in the treatment of several diseases like epilepsy, and shows first promising effects in cancer therapy. Molecular mechanisms, however, are still poorly understood. Within **NOTOX** we are studying effects of VPA using HepaRG cells in 2D and 3D culture.

Large Scale Multi-omics VPA Case Study Using 2D Cultivation

To gain more insight into the mechanisms occurring during the development of steatosis, we designed a two-week *in vitro* experiment using the HepaRG cell line together with VPA. A combination of '–omics' techniques from all levels, functional data and microscopical analysis, obtained at different stages during the development of steatosis gave rise to a huge data set,

which is currently subjected to an in-depth bioinformatics analysis. The experimental protocol and analyses for this experiment are summarised in *Figure 4.46*.

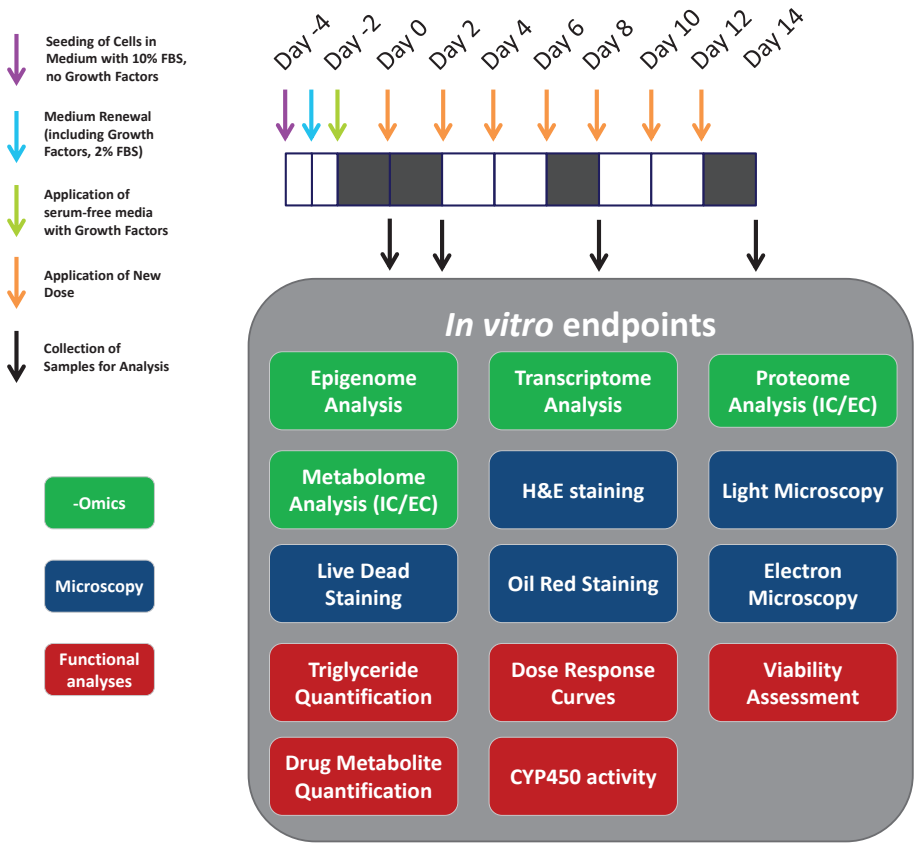


Figure 4.46 *Exposure protocol and in vitro endpoints used for the multi-omics study on VPA toxicity in 2D HepaRG cultures upon long-term repeated dose exposure.*

Viability of HepaRG cells during the two-week VPA treatment decreased in a dose- and time dependent manner. Treatment with concentrations below the c_{max} (0.29 mM), resulted in a decreased viability after 8 and 14 days.

Metabolic Characterisation. We found accumulation of lipids in HepaRG cells after two days of treatment with VPA (data not shown), which has previously been related to up-regulation of genes involved in synthesis of lipids and down-regulation of genes involved in lipolysis (*Wang et al., 2012*).

Treatment of HepaRG cells with VPA resulted in decreased uptake of glucose, followed by a decrease in secretion of lactate (*Figure 4.47*). Likewise, rates in the tricarboxylic acid (TCA) cycle and those of branched-chain amino acid (BCAA) degradation decreased. *Luis et al.* (*2007*) reported that VPA inhibits the dihydrolipoyl dehydrogenase, an essential part of the branched-chain α-ketoacid dehydrogenase complex and the pyruvate dehydrogenase complex. Furthermore, an indirect inhibition of the urea cycle has been observed upon treatment with VPA (*Aires et al., 2011*), which is consistent with the fluxes measured in our *in vitro* study.

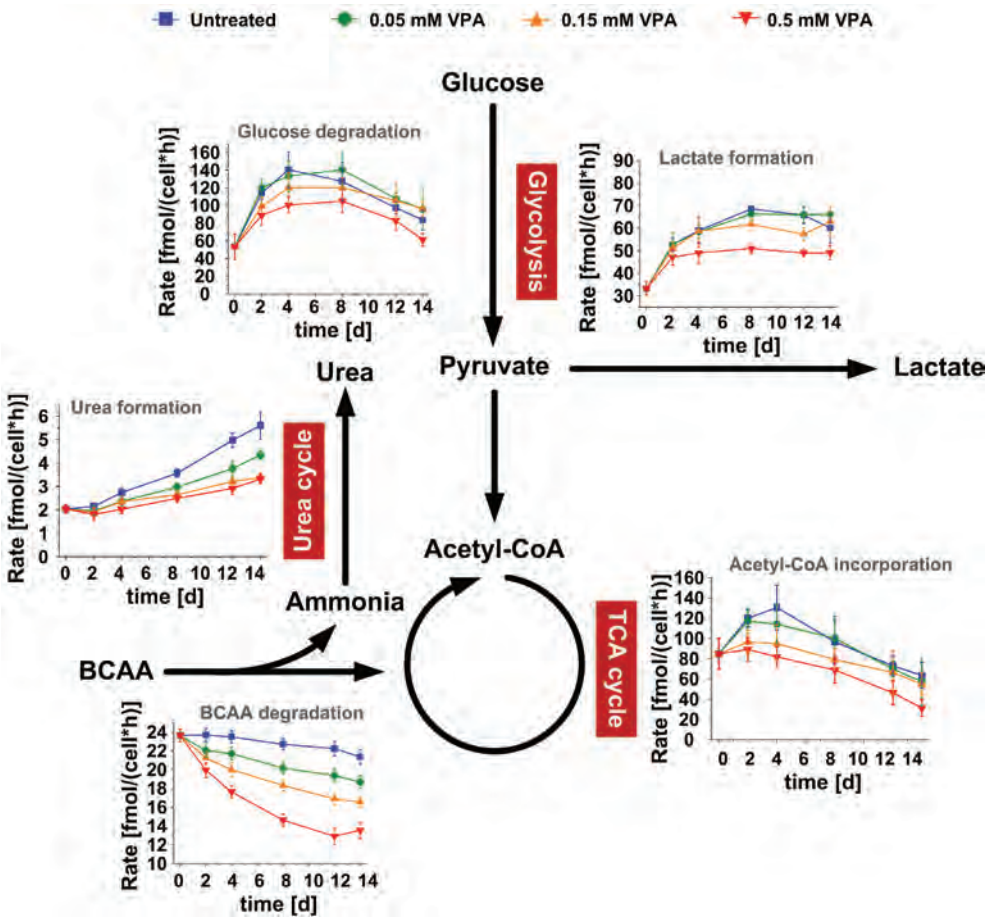


Figure 4.47 *Selected metabolic fluxes in the central carbon metabolism of HepaRG cells upon repeated dose treatment with VPA. A reaction occurring in the direction of the arrow is indicated by a positive sign. Error bars indicate standard deviation (n=3). BCAA = branched-chain amino acids.*

Transcriptomic Analysis. The RNA for transcriptomic studies was extracted using the AllPrep DNA/RNA/Protein kit (Qiagen). Samples for RNA extraction were obtained from cultures exposed to valproic acid (0, 0.05, 0.15 or 0.5 mmol/L) for 0, 2, 8 or 14 days. The VPA exposures were conducted in triplicates. All RNA samples had a high quality according to the RNA Integrity Number (RIN) of 8.9 or higher (RK6 ScreenTape®, Agilent Technology, Santa Clara, CA, US). Gene expression analysis was performed using Affymetrix GeneChip® Human Transcriptome Array (HTA) 2.0 at the Bioinformatic and Expression Analysis (BEA) core facility (Karolinska Institutet, Huddinge, Sweden). Differentially expressed genes were visualised using hierarchical clustering (TIGR Multiexperiment Viewer, MeV). Fold-changes (FC) were calculated from average of the triplicates.

Since more than 400 genes were up- or down-regulated in control cells as an effect of culture time, the effect of VPA treatment at each time point was compared to the corresponding control samples. Approximately 300 genes were found to be up- or down-regulated more than 1.5 folds (*Figure 4.48*). Based on a cluster analysis, the genes can be divided into 4 different groups.

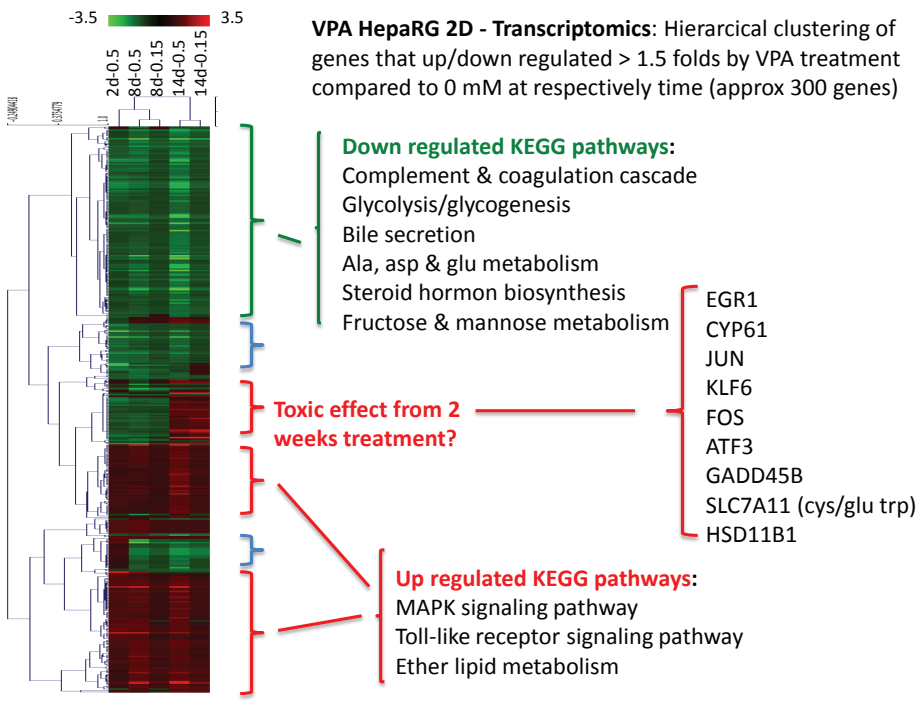


Figure 4.48 *Hierarchial clustering of FC at each time points compared to the corresponding control.*

Among the down-regulated genes the KEGG bile secretion pathway can be exemplified with the BSEP transporter, which is down regulated already after 2 days treatment with *0.15 mM VPA (Figure 4.49).*

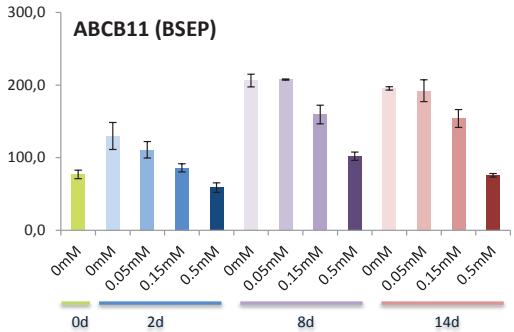
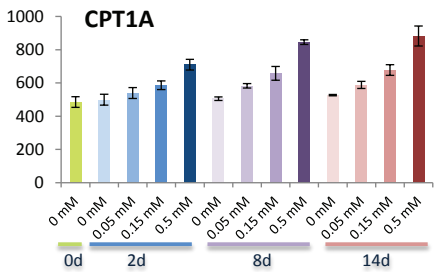


Figure 4.49 *Signal intensity of gene expression analysis of BSEP mRNA, average of triplicates ± SD.*

VPA enters the liver mitochondria with help of carnitine and carnitine palmitoyltransferase I (CPT1A). Long-term treatment with VPA will deplete carnitine and inhibit the CPT1A enzyme, resulting in a compensatory up-regulation of the CPT1A gene expression (*Figure 4.50A*). Interestingly, only one gene product, CYP4X1, was induced at all three time points by 0.15 mM VPA (*Figure 4.50B*). This isoform of cytochrome P450 is a PPARa inducible gene and suggested to be involved in lipid metabolism, which might be of relevance since the steatotic compound VPA is influencing the lipid homeostasis to a high degree.



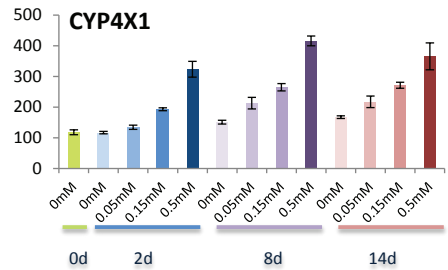


Figure 4.50 *Signal intensity of gene expression analysis, average of the triplicates ± SD. A: carnitine palmitoyl transferase 1 mRNA. B: CYP4X1 mRNA.*

In addition, VPA is also known to inhibit the histone deacetylase (HDAC) and osteopontin (SPP) mRNA which are known to be induced by HDAC inhibitors and found to be up-regulated

2-3 fold by VPA treatment (*Figure 4.51*). Osteoponin is an interesting pro-fibrogenic protein which has been shown to delay liver fibrosis resolution due to sustained fibrillary collagen-I disposition (*Leung et al., 2013*), but also been suggested to protect from early alcohol-induced injury (*Ge et al., 2014*).

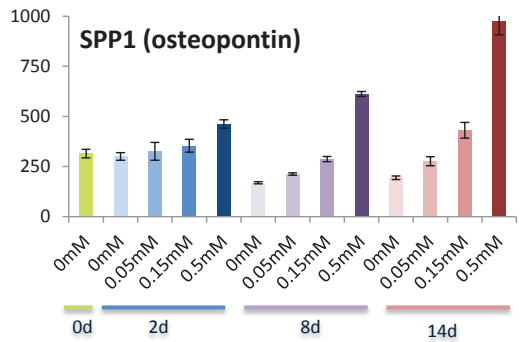


Figure 4.51 *Signal intensity of gene expression analysis of osteopontin mRNA, average of triplicates ± SD.*

In one of the groups of genes in *Figure 4.48* one can observe a considerable induction as a late response after 14 days of VPA treatment. Among these genes we identified several stress response genes, e.g. FOS, JUN, ATF3, which indicate a more severe toxic response after long-term VPA treatment of the HepaRG cells.

Unveiling drug-induced effects on the HepaRG proteome. Using a label-free proteomics approach including manual validation of more than 4000 peptide signals, 1300 proteins could be quantified across all 13 groups analysed with an intragroup coefficient of variation of less than 20%. Abundance of around 200 proteins changed significantly between all samples in a dose- and time-dependent manner, i.e. the longer and the more VPA was applied, the more proteins turned out to be significantly altered. Pathway analysis of the differential proteins indicated that especially lipid metabolism was affected by VPA-treatment, which is in accordance with the expected steatotic effects of VPA. Furthermore, the effects on the proteome were visible already when commonly applied endpoint assays like e.g. cell viability or ATP content did not yet indicate toxicity.

Epigenomic Profiling. A well-known function of VPA is to inhibit protein deacetylases including class I histone deacetylases (*Khan et al., 2008*). In the presented study, HepaRG cells were

used to study epigenetic effects of repeated long-term VPA treatment (up to 3 weeks). We performed genome-wide DNA methylation (Illumina 450K BeadChip array) and histone modification analysis (ChIP-seq) at different time points. Epigenetic data were also correlated with global gene expression data (Affymetrix).

In a first experiment 2D cultures of HepaRG were cultivated for 2, 8 and 14 days with repeated, every second day, treatment of 0.05 mM or 0.15 mM VPA, respectively. Analysing approx. 485.000 CpG positions in the HepaRG genome, no prominent effects on the DNA methylome were observed. Notably, only 38 genes showed differential gene expression (fold changes > 2) by cultivation with VPA treatment causing only slightly more changes.

As VPA is known to inhibit histone deacetylases, in a second experiment HepaRG cells were cultivated for 14 and 21 days to analyse the histone modifications H3K9ac and H3K4me3 using ChIP-seq. Preliminary bioinformatic analysis showed an overall decrease of normalised H3K9ac peaks during cultivation (*Figure 4.52A*), while H3K4me3 appears to be more stable (*Figure 4.52B*). This points to VPA blocking partially the loss of H3K9ac. Interestingly, also new H3K9ac and H3K4me3 peaks (*Figure 4.52A* and *B*) were detected at d14 and d21, among them sites that coincide with changes in gene expression. In *Figure 4.52C* and *D* we show the increase of H3K9ac and H3K4me3 upon cultivation and treatment, respectively, in the promoters of *VNN1* (pantetheinase, involved in liver lipid metabolism) and *CYP4A11* (member of the cytochrome P450 family) which goes along with increased gene expression. To prove the reliability of the results, a second treatment and ChIP-seq experiment was performed which is currently under evaluation.

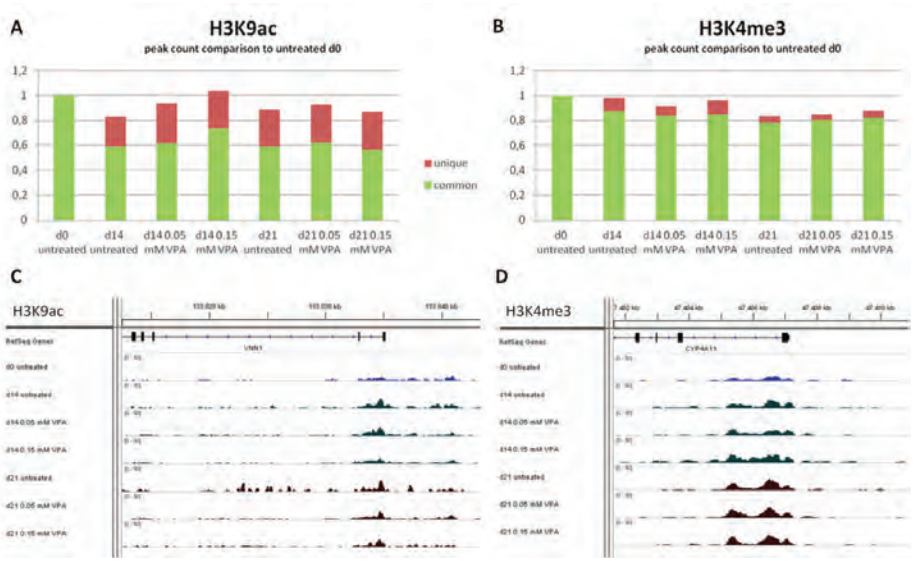


Figure 4.52 *Effects of long-term VPA treatment on histone modifications H3K4me3 and H3K9ac in HepaRG. A: Normalised H3K9ac peak comparison to d0 untreated control (peaks*

that occur in d0 and remain in all samples analyzed are displayed in green, sample-specific peaks in red); B: Normalised H3K4me3 peak comparison to d0 untreated control; C: IGV browser view of the VNN1 promoter regions with upregulated H3K9ac after cultivation and VPA treatment; D: IGV browser view of the CYP4A11 promoter region with upregulated H3K4me3 after cultivation and VPA treatment.

Valproic Acid Case Study – HepaRG Spheroids

Using the hanging drop method (GravityPLUS™, inSphero), we have previously shown that spheroids from human HepaRG cells can be formed yielding a more metabolically competent and liver-like organotypic *in vitro* culture system which can be maintained for a longer period of time. The HepaRG cell line consists of a 50/50 mix of hepatocytes (CYP3A4 positive) and cholangiocytes (CK19 positive) and we wanted to know whether this configuration would also remain stable during the period during which the repeated dosing of valproic acid (VPA) in the steatosis case study would take place. Using cryosectioning of the spheroids and subsequent staining with antibodies targeting CYP3A4 and CK19, we could show that the distribution of the cell type remains constant and mixed during the extended culture period (*Figure 4.53*).

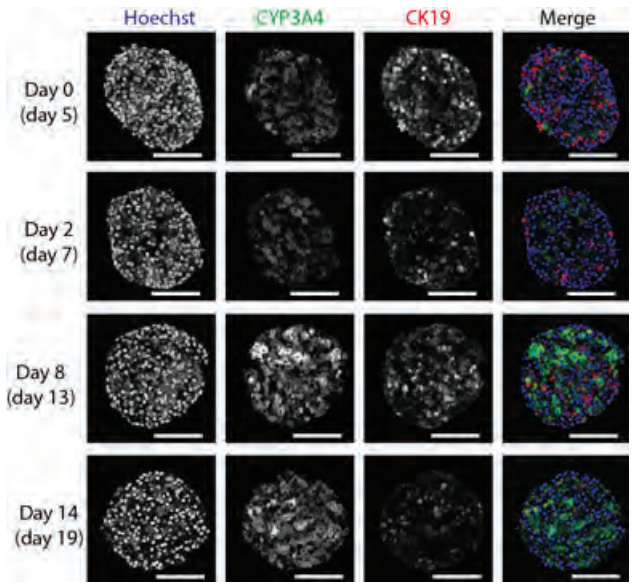


Figure 4.53 *HepaRG cell types remain equally distributed throughout an extended culture period. HepaRG sperhoids were fixated, frozen and cryosectioned at different time-periods following seeding (in parenthesis) and spheroid formation. Presence of hepatocytes and cholangiocytes was determined by immunostaining for CYP3A4 (green) and CK19 (red) respectively. Representative images of several spheroids are shown and the white bar indicates 100 µM.*

Using this system we then demonstrated increasing toxicity induced by VPA by extending the dosing of the drug from 2 days to 8 and 14 days, decreasing the IC_{50} values from 7 mM after 2 days to 1.5 and 0.6 mM respectively (*Figure 4.54A*). Moreover, the stability/reproducibility of the HepaRG spheroid system for toxicity testing was shown by the small difference in the dose response curves when repeated for 14 days in two independent experiments (*Figure 4.54B*).

A

B

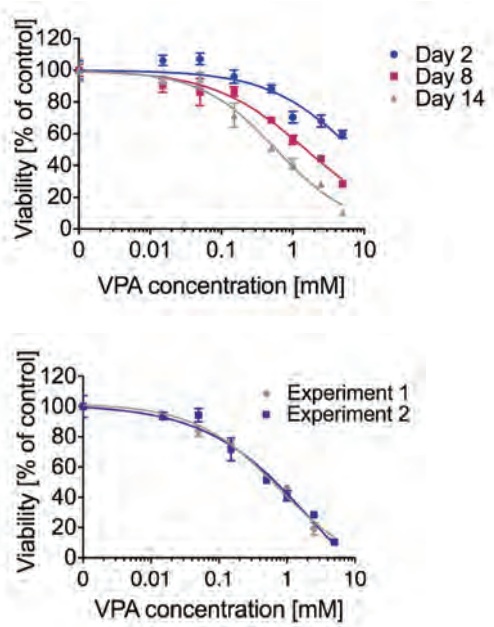


Figure 4.54 *VPA reproducibly induces enhanced toxicity in HepaRG spheroids with repeated exposure. HepaRG spheroids were dosed every second day with indicated concentrations of VPA and the viability was determined by measuring the ATP levels at the shown time-points. A – viability at different time point, B – reproducibility at 14d. Data presented are means ± S.E.M. of 3-5 spheroids per data point.*

The toxicity of VPA has been linked to many intracellular events leading to the accumulation of lipids and thereby steatosis. Among these, induction of oxidative stress and inhibition of the enzyme carnitine palmitoyl transferase 1A (CPT1A) have been proven important. A sign of oxidative stress is the depletion of glutathione, one of the two major intracellular antioxidant systems. Interestingly for this 3D organotypic culture system, this could be observed already after 2 days exposure to VPA at a non-toxic concentration of 0.5 mM (*Figure 4.55A*). As a result of the inhibitory effect VPA has on CPT1A, a compensatory increase in its gene expression has been reported. In the HepaRG spheroids, a concentration and time dependent increase in the RNA expression of CPT1A could be observed accordingly (*Figure 4.55B*).

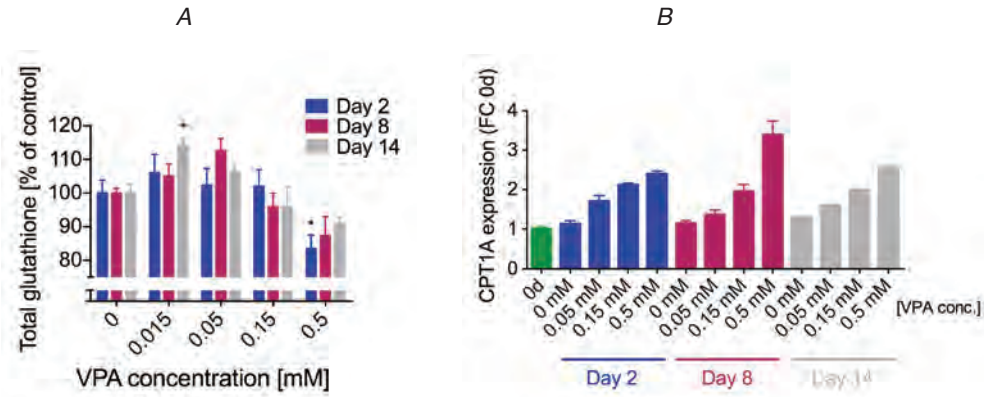


Figure 4.55 *VPA induces glutathione depletion and CPT1A expression in a concentration and time-dependent manner. (A) The total amount of glutathione was determined using the GSH/GSSG-Glo® assay from Promega and data presented are means from 3-4 spheroids ± S.E.M.; * indicates significance at a level of $p < 0.05$ determined by 2-way ANOVA. (B) Carnitin palmitoyl transferase (CPT1A) expression was determined by qPCR after VPA exposure to indicated concentrations and time periods. Data presented are means of duplicate samples.*

These data illustrate the applicability of this organotypic 3D system for studying the toxic effects of VPA and we are currently investigating more in-depth mechanisms behind VPA-induced hepatotoxicity using proteomics, metabolomics as well as electron microscopy.

Coupled physiologically based pharmacokinetic (PBPK) and cellular mechanism of action modelling

The **NOTOX** partner INSIL uses in-house validated whole body physiologically-based pharmacokinetic (PBPK) models. These models predict local organ and tissue concentration dependent on the administration route and dose. The human PBPK model was coupled to cellular hepatic valproic acid metabolism and toxic mechanism of action model (Figure 22). In brief, the PBPK model includes the distribution into 16 different organs, different administration routes, e.g. peroral, gastric absorption mechanisms, and renal and hepatic clearance (*Figure 4.56*, left). The hepatic clearance is in this case replaced by specific VPA metabolism (*Figure 4.56*, right).

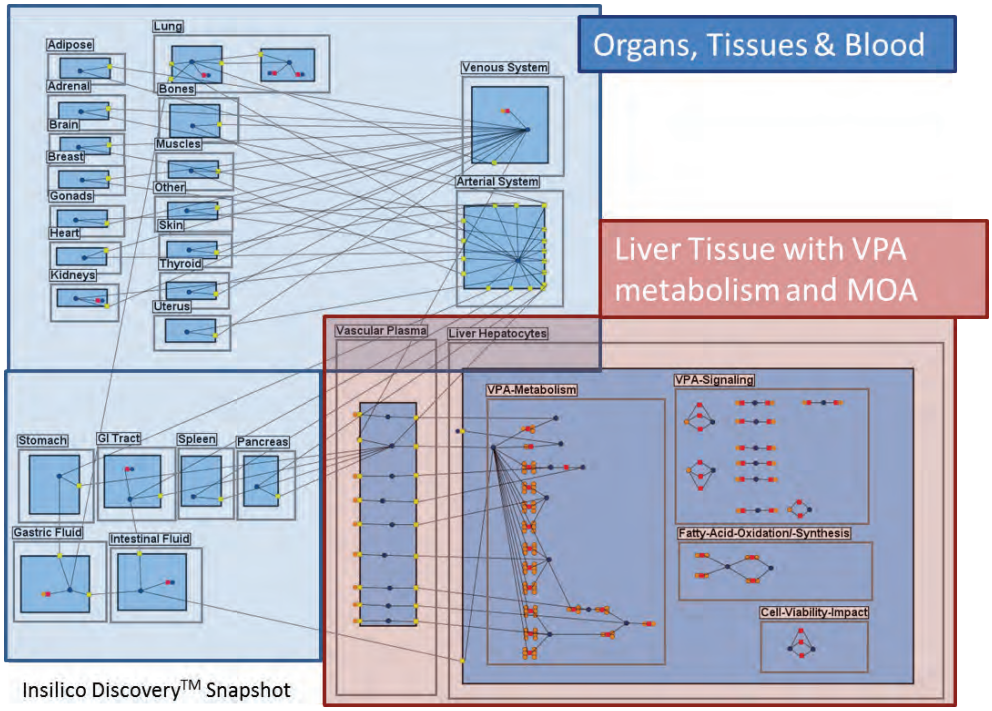


Figure 4.56 *Scheme of coupled PBPK and hepatic metabolism and mechanism of toxic action model of VPA. The cellular VPA model is coupled to the PBPK model via blood influx from the arterial pool into the liver vascular compartment and efflux to the venous pool. The cellular valproic acid network includes metabolism and toxicokinetic mechanism of actions.*

The VPA metabolism model includes CYP- and beta-oxidation, glucuronidation, and CoA-conjugations, similar as described in the literature (*Ghodke-Puranik et al., 2013*).

Toxic mechanisms of actions of VPA implemented in the model include the attenuation of cell viability via acute effects by the toxic metabolite 2,4-diene-VPA, and via acute and long-term impact on the lipid metabolism. The acute impact on lipid turnover includes the beta-oxidation of VPA that is competing with fatty acid metabolism, leading to increased fatty acid accumulation, whereby the long-term impact on lipid metabolism considers the up-regulation of genes in fatty acid synthesis and down-regulation of genes in fatty acid metabolism via induction of sterol regulatory element-binding protein (SREBP) and the inhibition of peroxisome proliferator-activated receptor alpha (PPARalpha; *Wang et al., 2012*), respectively. Finally, this leads to reduced cell viability and potential hepato-steatotic outcome *in vivo*.

The PBPK model was validated against *in vivo* data describing the pharmacokinetic plasma profiles of the parent compound after a single dose in human (*Mihaly et al., 1979*). Mechanism

and mode of action parameters were pre-set, some from the literature (*Argikar & Remmel, 2009; Kiang et al., 2006*), to hypothetically display toxic VPA effects from the dynamics in plasma and hepatocytes' uptake of VPA to the potential accumulation of triacylglycerols and cell viability impact. This test scenario is physiologically not realistic due to much lower than maximally recommended VPA dose-levels (<< 4.8 g/day), but supports the identification, if the model can describe the expected toxic mechanism cascade qualitatively (*Figure 4.57*). Thus, fatty acid (FA) and triacylglycerol (TAG) accumulation can be simulated (*Figure 4.57*, left). Together with the synthesis of a toxic VPA-metabolite and a competitive fatty acid metabolism inhibitor metabolite (*Figure 4.57*, upper right), time-dependent cell viability reduction (*Figure 4.57*, lower right) is described by the model.

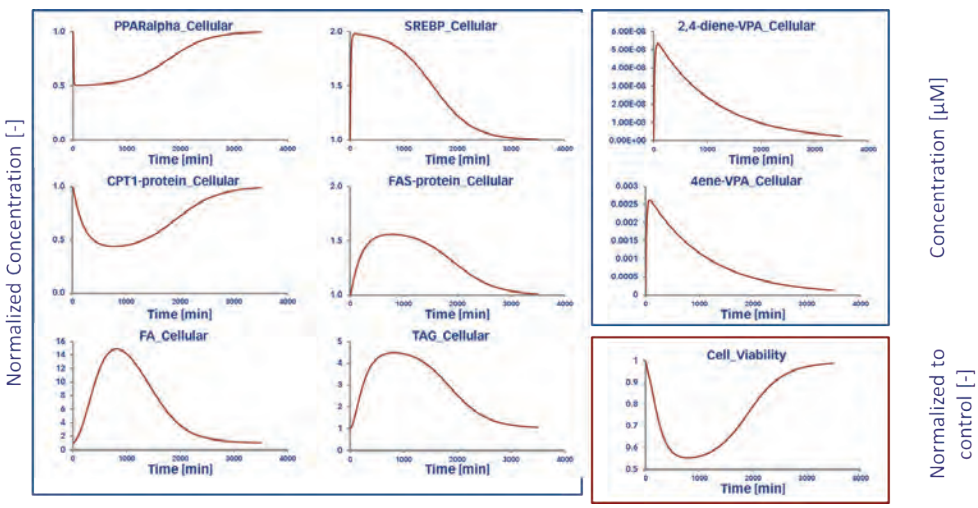


Figure 4.57 *Qualitative description of VPA-triggered toxicokinetic profiles. Displayed are theoretical profiles of (i) cellular signalling compounds (PPARalpha, SREBP), leading to downregulated expression of enzyme CPT1 and upregulated expression of enzyme FAS, contributing to fatty acid (FA) and triacylglycerol (TAG) accumulation (left side, blue box). Further, (ii) theoretical dynamic profiles of VPA metabolite 2,4-diene-VPA (causing oxidative stress) and 4ene-VPA (competitive inhibitor of fatty acid metabolism) are shown (right side, upper blue box). Both effects contribute to potentially reduced cell viability (right side, lower red box).*

In the next steps, the toxicity related mechanism of action model parameters will be identified on the basis of experimental data obtained in VPA long-term experiments covering time-series measurements of metabolites, transcripts and proteins. The metabolite measurements from **NOTOX** partners and from Mario-Negri-Institute (ToxBank) support the identification of VPA metabolism. The protein data will help to identify the signalling network and dynamics.

Transcript data will support the identification of regulatory steps. The technological approach of coupling signalling pathway and metabolic network models has been recently established by **NOTOX** partner INSIL in cooperation with partners from the group of Dr.-Ing. Steffen Klamt, Max-Planck-Institute for Dynamics of Complex Technical Systems Magdeburg (*Ryll et al., 2014*). Similar kinetics were implemented in the VPA-effected signalling to fatty acid metabolism steps.

A detailed description of VPA-MoA-modelling can be found in the extended abstract of the fifth **SEURAT-1** Annual Meeting poster award for the poster entitled 'Coupled Modelling of PBPK and Toxic Mechanisms of Action of Valproic Acid in Liver' (see section 4.9.3.2).

4.6.4 Contributions to the SEURAT-1 Case Studies

NOTOX has put a lot of effort in the VPA case study that was carried out in close cooperation of most partners of the consortium (see above). Besides that, the **NOTOX** consortium has performed an extensive study with bosentan as a standard reference compound for investigating cholestasis, which will certainly add value to the **SEURAT-1** case study focussing on the adverse outcome pathway construct from bile salt export pump inhibition to cholestatic injury (see section 3.3.1) and will be reported in the following.

Effects of repeated dose bosentan exposure on the carbon metabolism of 2D and 3D HepaRG cultures in a two-week study

3D hepatocyte cultures more and more replace conventional 2D cultivation due to advantages such as improved metabolic competence, long-term viability and *in vivo*-like cellular environment including cell-cell contacts. In this study, **NOTOX** investigated the sensitivity of different HepaRG cultures (2D cultures and 3D spheroids) to repeated bosentan exposure for two weeks and whether a different culture environment can result in different regulation in the central carbon metabolism upon drug exposure. We found that 3D HepaRG cultures were significantly more susceptible to bosentan-induced cytotoxicity, compared to 2D cultures with an EC_{50} value of 20 μM in the 3D and 120 μM in the 2D case.

Upon treatment with concentrations around the EC_{10}, sub-toxic concentrations and the c_{max}, we observed significant metabolic changes that were clearly different in 2D and 3D cultures (e.g. lactate; *Figure 4.58*). In many cases, alterations were in the opposite direction (e.g. glucose). The mass isotopomer distribution (MID) in lactate upon bosentan exposure and incubation with ^{13}C substrate ([1,2]-^{13}C-glucose, U-^{13}C-glutamine) showed accumulation of labelled carbon atoms, when treated with concentrations around the EC_{10} (data not shown). This indicates increased conversion of either glucose or glutamine, or even both substrates, to lactate. Previous studies have shown that upon bosentan exposure carbon metabolism is influenced via the nuclear receptors farnesoid X receptor (FXR) and peroxisome proliferator-

activated receptor α (PPARα), however little is known on differences in expression of these proteins in 2D and 3D culture systems (*Zollner & Trauner, 2009*). Beside differences in nuclear receptor expression in 2D and 3D cultures, metabolic differences might as well be based on different levels of stored polymers, such as glycogen, allowing cells to react differently on external stimuli. In conclusion, 3D spheroid HepaRG cultures are significantly more sensitive to repeated bosentan exposure than 2D cultures. Elucidation of the cause for differences in metabolic responses in 2D and 3D cultures is under further investigation.

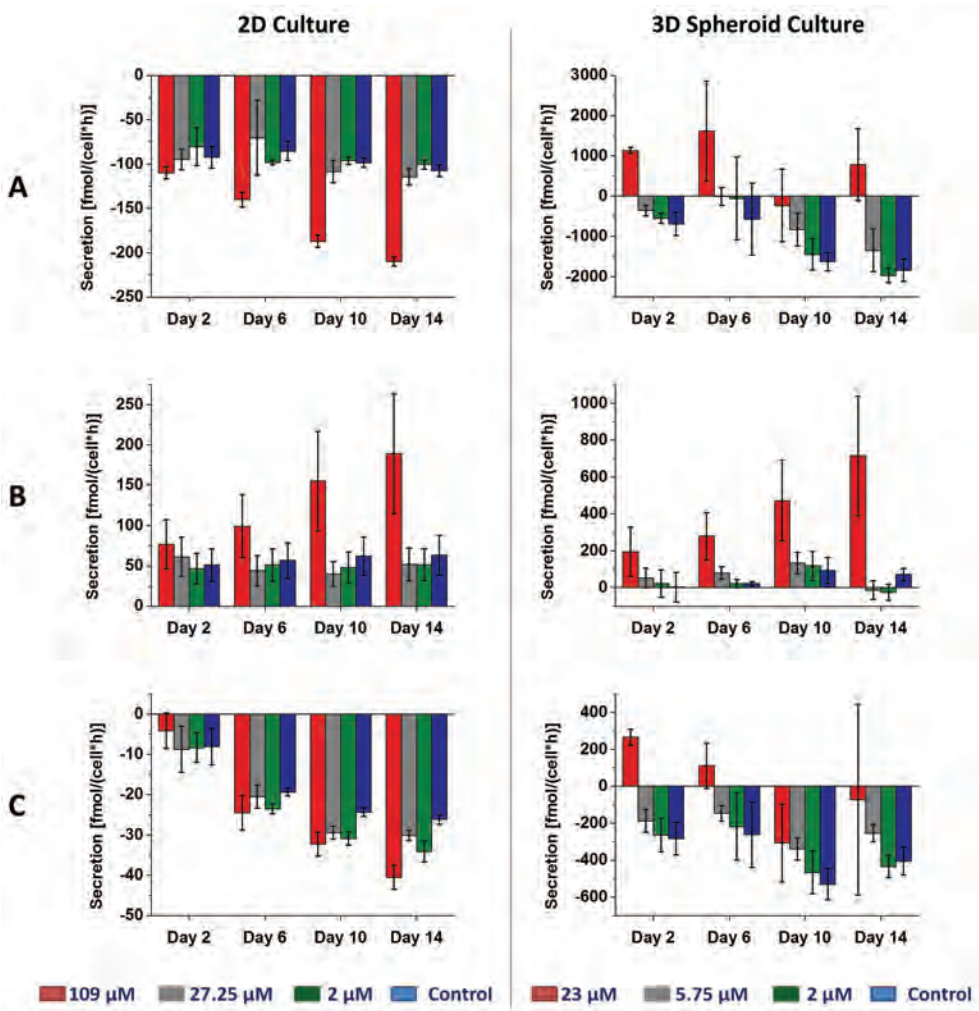


Figure 4.58 *Selected secretion rates of 2D cultures and HepaRG spheroids in the course of repeated bosentan exposure. A, B and C represent rates for glucose, lactate and glutamine respectively. Conditions shown are* EC_{10} *(red), a subtoxic concentration (grey) and* c_{max} *(green) in addition to the untreated control (blue). Error bars indicate standard deviation (n = 3).*

Improvement of the organotypic cell culture system – Introducing bile acid co-exposures for the identification of cholestatic compounds

Hepatic cholestasis is the most common reason for the manifestation of drug-induced acute liver failure and it is therefore desired to identify drugs/drug candidates with a cholestatic potential before they reach the market. Others have shown that this can be achieved by co-exposing primary rat and human hepatocytes to drugs and a concentrated mix of bile acids. Here we have tested the same approach in a more 'liver-like' culture setting by using the HepaRG 3D spheroid system. For this purpose a mix of six different human bile acids was used at different concentrations according to which they can be found in human plasma. To define the highest concentration at which no toxicity could be seen, we first titrated the bile acid mix, which resulted in determining a 50-fold higher concentration of the normal human plasma concentration as the highest non-toxic concentration after 8 days repeated exposure (every second day; *Figure 4.59A*). Important for more chronic exposures, this bile acid concentration remained non-toxic after 14 days of repeated dosing (*Figure 4.59B*).

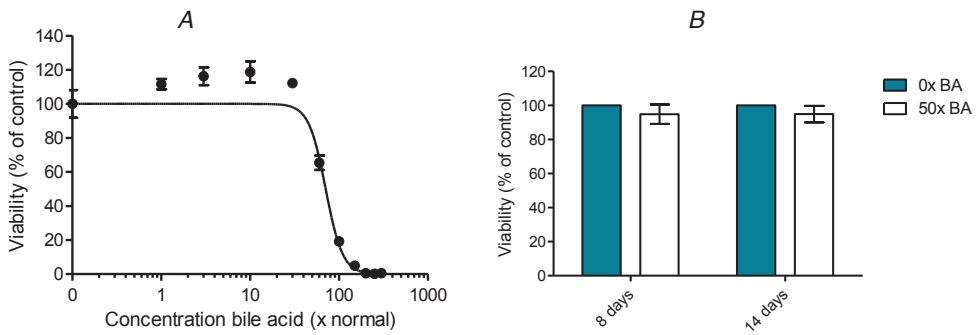


Figure 4.59 *Bile acid induced toxicity in HepaRG spheroids. (A) HepaRG spheroids were dosed every second day for 8 days with a concentrated bile acid mix at the indicated concentrations times normal plasma concentrations. Data presented are means from 4-5 spheroids ± S.E.M. and IC90 was defined as 50-fold higher of the normal bile acid concentration. This concentration was also not toxic after 14 days of repeated exposure (B) were presented data are means of 3 independent experiments ± S.E.M.*

We subsequently assessed the toxicity of three cholestatic compounds; chlorpromazine, troglitazone and bosentan as well as two non-cholestatic compounds; paracetamol and tetracycline during 14 days of repeated exposure with and without the 50 times concentrated bile acid mix (*Figure 4.60*). We could thereafter conclude that only the toxicity of the cholestatic compounds in the HepaRG spheroid model could be enhanced with the non-toxic bile acid mix, demonstrating the specificity of the system. We are currently investigating the mechanism behind the bile acid enhanced toxicity of the cholestatic compounds to further validate the system.

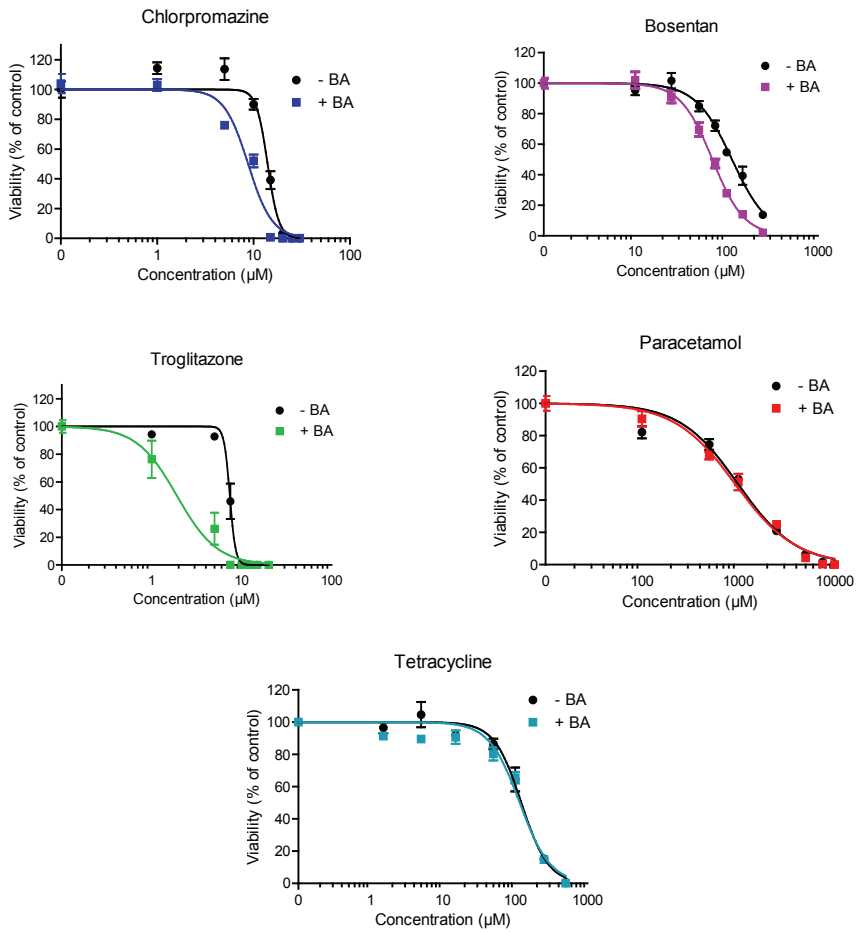


Figure 4.60 *Bile acids enhance the toxicity of cholestatic but not non-cholestatic compounds in HepaRG spheroids. HepaRG spheroids were dosed every second day for 14 days with cholestatic (chlorpromazine, troglitazone and bosentan) and non-cholestatic compounds (paracetamol and tetracycline) with and without the addition of 50 times concentrated bile acid mix. Viability was assessed by measuring ATP and data presented are means from 4-5 spheroids ± S.E.M.*

4.6.5 Cross-Cluster Cooperation

Various cross-cluster collaboration possibilities were identified and discussed during the **SEURAT-1** Annual Meetings in 2014 and 2015 (Barcelona, Spain). Collaboration with ***HeMiBio*** using HepaRG co-cultures with human stellate cells has been started and joint results were presented at the **SEURAT-1** Annual Meeting in 2015. Furthermore, **NOTOX** has started work using stem cell-derived cardiomyocytes from *SCR&Tox*.

In addition, the collaboration with COSMOS regarding PBPK modelling was continued and joint results were presented at the **SEURAT-1** Annual Meeting in 2015. The collaboration with ToxBank was continued as well and will 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** 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, as reported in the previous sections 4.6.3 and 4.6.4, the **NOTOX** consortium contributes actively to the **SEURAT-1** case studies. The **NOTOX** coordinator participated in the **SEURAT-1** *ab initio* case study workshop on 9-10 October 2014, which was organised by the European Commissions Joint Research Centre in Ispra, Italy.

The **NOTOX** consortium has been active at joint **SEURAT-1** events and contributed the six following posters to the poster session at the **SEURAT-1** Annual Meeting in Barcelona in March 2014:

Cellière, G., van Liedekerke, P., Bucher, J., Klein, S., Niklas, J., El-Kariem, S., Reif, R., Noor, F., Hengstler, J.G., Mauch, K., Heinzle, E., Drasdo, D.: Modelling spheroid formation and *in vitro* toxicity aiming at toxicity simulations *in vivo*

Reif, R., Günther, G., Ghallab, A., Widera, A., Cellière, G., van Liedekerke, P., Friebel, A., Drasdo, D., Heinzle, E., Hengstler, J.G.: Structure modelling – comparing spheroids with the *in vivo* situation.

Klein, S., Mueller, D., Tascher, G., Tierling, S., Johansson, I., Bucher, J., Shevchenko, V., Maggioni, S., Lutsik, P., Bertile, F., Niklas, J., Chesné, C., Peters, P., Walter, J., Ingelmann-Sundberg, M., van Dorsselaer, A., Heinzle, E., Noor, F.: Case Study: Multi-omics study on long-term repeated dose toxicity of valproic acid with steatosis as mechanism of action.

Klein, S., Maggioni, S., Bucher, J., Niklas, J., Mauch, K., Heinzle, E., Noor, F.: Biokinetics and metabolome analysis on HepaRG cells upon bosentan exposure for the assessment of repeated dose toxicity.

Fredriksson, L., Müller, D., Schweitzer, V., Persson, A., Virding, S., Johansson, I., Klein, S., Heinzle, E., Noor, F., Ingelman-Sundberg, M.: Studying repeated dose toxicity of valproic acid (VPA) in a HepaRG 3D system.

Johansson, I., Mueller, D., Fredriksson, L., Klein, S., Noor, F., Ingelman-Sundberg, M., Heinzle, E.: Toxicity of valproic acid as revelaed from experiments in 2D and 3D HepaRG systems.

4.6.6 Expected Progress within the Fifth Year

Multi-omics experiments: Further experiments with other selected compounds, e.g. bosentan, chlorpromazine are planned and executed. These experiments will be carried out by partners University of Saarland and Karolinska Institutet in joint collaboration and with selected '-omics' analyses.

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. Proteomic analysis of spheroids will be further refined aiming at the analysis of only few spheroids. Different endpoints will be used depending on the study compound. HepaRG spheroids will be compared to similar spheroids generated from 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. Tomato shall be applied in HepaRG as cellular membrane marker to improve 3D image analysis especially for MRP2 activity studies using HepaRG spheroids.

Agent-based spheroid modelling: 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. Furthermore, the spatio-temporal modelling of spheroid formation in the hanging drop setting will be continued to gain insights into the spheroid architecture. The existing model will be extended from the *in vitro* monolayers and spheroids to *in vivo* liver lobules. The intracellular toxicity models 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.

IVIVE using PBPK based modelling: The modelling efforts will be intensified as results from VPA and other experiments are now extensively available. PBPK modelling will be used in close collaboration with the COSMOS project.

4.6.7 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 next generation sequencing methods promoting epigenomic, transcriptional analyses enormously, are developing at a very high rate 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. The structuring and harmonisation of adverse outcome pathways (AOPs) on an international level will soon strongly support pathway oriented mathematical models that will lead to improved *in vivo in vitro* extrapolation using PBPK based modelling. Even more, a systems biology approach involving multiscale 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 less and less limited in their applicability due to the lack of sufficiently sensitive analytical techniques, e.g. for metabolome analysis. 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 IVIVE of long-term toxicity prediction in humans, 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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4.7 ToxBank: Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology

ToxBank

4.7.1 Introduction and Objectives

ToxBank is the cross-cluster infrastructure project whose activities support the collaborative research activities of all **SEURAT-1** partners and consortia. To that end, **ToxBank** has established a dedicated web-based warehouse for toxicity data management and modelling; a 'gold compound' database and repository of selected test compounds 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.

4.7.2 Main Achievements and Challenges in the Fourth Year: ToxBank Support Services

Based on a detailed requirements gathering exercise covering uploading and searching as well as an integrated data analysis, **ToxBank** has developed tools and support mechanism/processes for the population and use of the information generation of **SEURAT-1** into its data warehouse. In autumn 2014 the support of public access to both upload and access **ToxBank** data was launched.

Most **ToxBank** data warehouse support operations, including uploading of protocols and other documents as well as searching and downloading records, are implemented with a combination of on-line and face-to-face meetings, as well as through self-help material available from the **ToxBank** website. The preparation and upload of formatted data based on the ISA-tab universal data exchange format can be complex and **ToxBank** has developed processes to assist in this preparation. This process is usually performed in collaboration with the data generators.

Support Material

A variety of training and on-line resources have been developed to support the use of the ToxBank data warehouse. These resources (available under www.toxbank.net) include a guideline for the generation of protocols, a user guide for describing how the different **ToxBank** features can be used as well as an extensive collection of video tutorials outlining how to prepare the data, how to upload documents and data, how to search and download **ToxBank** content as well as background information on these activities.

Compliance

It is the responsibility of the project investigators that their experiments are well-documented and the data is entered in a quality manner. **ToxBank** provides support for these compliance aspects through the use of guideline documents as well as via tools. The process of uploading the data with the ISA-tab universal data exchange format ensures that the experiment is fully characterised including the characterisation of the biological material and experimental factors, all of which are linked to appropriate ontology terms. The incorporation of protocols for defining all steps supports compliance with full experimental documentation.

Sustainability

The **ToxBank** consortium agreed to the sustainable maintenance of access to all **ToxBank** reference data generated on the **SEURAT-1** programme as an OpenTox resource through at least 2020.

4.7.3 Selected Highlight: ToxBank Integrated Analysis

Assay data such as from ToxCast or other sources may be used in combination with '-omics' data and background knowledge to strengthen examination of the mechanistic weight of evidence in heterogeneous data, both in strengthening mechanistic or biomarker hypotheses, or negating signals e.g., non-functional '-omics' fold changes. Public data from sources such as Open TG-GATEs, ToxCast and PubChem can hence be combined with **SEURAT-1** data supporting integrated analysis, AOP development and case study risk assessments.

This examination of current model validity may be used to suggest most promising experiments for a next round of knowledge creation, e.g., a promising biomarker gene from '-omics' verified from a gene/protein pathway assay can be used as the basis to suggest qt-PCR and reporter assay to validate the expression and function of that gene, at least *in vitro*.

The additional issue of *in vivo* validation including *in vitro* - *in vivo* kinetics modelling for extrapolation is an important one, addressed elsewhere in this volume (see section 4.10.7). Here we describe a case study analysis of valproic acid (VPA) performed by **ToxBank** partners. VPA is one of the **ToxBank** standard reference compounds for liver toxicity, considered and used by a variety of **SEURAT-1** partners. VPA is widely used as a drug and hence has a rich background of knowledge in humans. VPA and its analogues also provide the category for several of the current case studies of **SEURAT-1** (see sections 3.4.2 and 3.4.3), and is a reference compound for the *ab initio* risk assessment case study (see section 3.5.2).

Valproic Acid

Valproic acid (VPA, valproate), an acidic chemical compound, has found clinical use as an anticonvulsant and mood-stabilising drug, primarily in the treatment of epilepsy, bipolar disorder and prevention of migraine headaches. VPA is a liquid at room temperature, but it can be reacted with a base such as sodium hydroxide to form the salt sodium valproate, which is a solid. Its primary use in medicine is in the treatment of epilepsy, bipolar mania and migraine prophylaxis. Recently, it has been trialed in the treatment of HIV and cancer, owing to its histone deacetylase-inhibiting effects. VPA is a low-molecular-weight (144.21) branched-chain carboxylic acid (pKa = 4.8).

Adverse Effects

The most common adverse effects of valproic acid are digestive complaints like diarrhea, nausea, vomiting and indigestion; vision problems like seeing double or lazy eye; hormonal disturbances (increased testosterone production in females and menstrual irregularities), hair loss, memory problems, weight gain, infections, low platelet count (which can make one bleed more easily), dizziness, drowsiness, tremor and headache. Less common, yet serious side

effects include liver damage, brittle bones (becomes far more common with long-term use), polycystic ovaries, movement disorders (which may be irreversible like tardive dyskinesia), psychiatric/neurologic disturbances like hallucinations, anxiety and confusion; swollen pancreas, low body temperature and potentially life-threatening blood abnormalities.

Toxicity

As a fatty acid analogue, VPA is a competitive inhibitor of fatty acid metabolism, which accounts for steatosis. It is also hepatotoxic by a mechanism that has not been resolved; however, this hydrophobic compound is used at very high concentrations and its promiscuous activity at these concentrations is likely due to disruption of membrane integrity. P450 ω-oxidation produces a reactive alkylating and free radical-propagating agent that adds to the toxicity profile.

This compound was selected as a **ToxBank** reference standard for steatosis via inhibition of ß-oxidation.

Metabolism

Valproate is metabolised almost entirely by the liver. In adult patients on monotherapy, 30-50% of an administered dose appears in urine as a glucuronide conjugate. Mitochondrial oxidation is the other major metabolic pathway, typically accounting for over 40% of the dose. Usually, less than 15-20% of the dose is eliminated by other oxidative mechanisms. Less than 3% of an administered dose is excreted unchanged in urine.

Mode of Action

The mechanism of action of VPA has not been established; however, it is thought to be related to a direct or secondary increase in concentrations of the inhibitory neurotransmitter, gamma aminobutyric acid (GABA) possibly caused by its decreased metabolism or decreased re-uptake in brain tissues. VPA prolongs the recovery of inactivated sodium channels. These properties may be responsible for its action as a general CNS depressant. VPA also alters fatty acid metabolism with impaired mitochondrial beta-oxidation and disruption of the urea cycle and can cause hyperammonemia, hepatotoxicity, metabolic perturbations, pancreatitis, cerebral edema, and bone marrow depression. Some of these effects may be associated with carnitine deficiency.

Analysis

We selected the medium concentration 24 hours TG-GATESs (http://toxico.nibio.go.jp/) processed '-omics' data set and performed an enrichment analysis with InCroMAP (*Wrzodek et al., 2012*) to provide the set of processed data shown in *Table 4.3* (using a filter of fold

changes (FCs) with a log2 absolute value greater than 0.5). The top two pathways affected were oocyte meiosis (6/298 genes, p-value = 0.0028, see *Figure 4.61* for perturbed pathway visualisation) and cell cycle (7/298 genes, p-value = 0.0039, see *Figure 4.62*) showing an impact by VPA on the cell cycle replication process. Numerous other pathways involving metabolism and fatty acid processing were affected with p-values of less than 0.05.

Table 4.3 *Results of the enrichment analysis to Kegg pathways with InCroMAP.*

#	ID	Name	List ratio	BG ratio	P-value	Q-value	Genes/Compounds
1	path:hsa04114	Oocyte meiosis	6/300	73/14867	2.859E-3	0.1646	CDC20, PLK1, IGF1, CCNE1, SGOL1, AURKA
2	path:hsa00140	Steroid hormone biosynthesis	4/300	33/14867	3.71E-3	0.1646	HSD17B2, CYP3A7, 1588, SULT1E1
3	path:hsa04110	Cell cycle	7/300	105/14867	4.084E-3	0.1646	CDC20, ORC6, PLK1, TGFB1, CCNE1, TTK, CDC25A
4	path:hsa00830	Retinol metabolism	4/300	36/14867	5.027E-3	0.1646	RDH16, CYP2C19, CYP4A11, CYP3A7
5	path:hsa00330	Arginine and proline metabolism	4/300	42/14867	8.464E-3	0.2218	ARG1, CPS1, OAT, PRODH2
6	path:hsa04975	Fat digestion and absorption	3/300	26/14867	0.0133	0.2902	MOGAT3, MOGAT2, MTTP
7	path:hsa05020	Prion diseases	3/300	29/14867	0.0176	0.3046	C6, HSPA5, C9
8	path:hsa00591	Linoleic acid metabolism	2/300	11/14867	0.0186	0.3046	CYP2C19, CYP3A7
9	path:hsa00380	Tryptophan metabolism	3/300	32/14867	0.0225	0.327	DDC, ACMSD, KMO
10	path:hsa00590	Arachidonic acid metabolism	3/300	34/14867	0.026	0.3409	CYP2U1, CYP2C19, CYP4A11
11	path:hsa04913	Ovarian steroidogenesis	3/300	39/14867	0.0359	0.4278	HSD17B2, IGF1, 1588
12	path:hsa00982	Drug metabolism - cytochrome P450	2/300	18/14867	0.0449	0.4295	CYP2C19, CYP3A7
13	path:hsa00062	Fatty acid elongation	2/300	19/14867	0.0492	0.4295	ELOVL7, ELOVL4
14	path:hsa01230	Biosynthesis of amino acids	3/300	45/14867	0.0494	0.4295	PKLR, ARG1, CPS1
15	path:hsa04621	NOD-like receptor signaling pathway	3/300	47/14867	0.0542	0.4295	CCL2, CARD6, TRIP6
16	path:hsa04918	Thyroid hormone synthesis	3/300	47/14867	0.0542	0.4295	PDIA4, HSPA5, ASGR1
17	path:hsa03430	Mismatch repair	2/300	21/14867	0.058	0.4295	MSH6, EXO1
18	path:hsa00983	Drug metabolism - other enzymes	2/300	22/14867	0.0625	0.4295	XDH, CYP3A7
19	path:hsa04974	Protein digestion and absorption	3/300	52/14867	0.0668	0.4295	ACE2, SLC3A1, SLC7A9
20	path:hsa04141	Protein processing in endoplasmic reticulum	5/300	130/14867	0.0748	0.4295	PDIA4, CRYAB, HERPUD1, HSPA5, DDIT3
21	path:hsa00232	Caffeine metabolism	1/300	4/14867	0.0783	0.4295	XDH
22	path:hsa00980	Metabolism of xenobiotics by cytochrome P450	2/300	26/14867	0.0811	0.4295	CYP2C19, CYP3A7
23	path:hsa05204	Chemical carcinogenesis	2/300	26/14867	0.0811	0.4295	CYP2C19, CYP3A7
24	path:hsa04914	Progesterone-mediated oocyte maturation	3/300	58/14867	0.0826	0.4295	PLK1, IGF1, CDC25A
25	path:hsa04976	Bile secretion	3/300	58/14867	0.0826	0.4295	SLC10A1, 9971, SLC22A1
26	path:hsa04115	p53 signaling pathway	3/300	59/14867	0.0852	0.4295	IGF1, CCNE1, SESN3
27	path:hsa01200	Carbon metabolism	3/300	63/14867	0.096	0.4659	PKLR, CPS1, G6PD
28	path:hsa05146	Amoebiasis	3/300	65/14867	0.1014	0.4745	ARG1, TGFB1, C9
29	path:hsa05215	Prostate cancer	3/300	69/14867	0.1122	0.5015	PDGFRB, IGF1, CCNE1
30	path:hsa04146	Peroxisome	3/300	70/14867	0.1148	0.5015	HAO2, XDH, ACOX2
31	path:hsa04151	PI3K-Akt signaling pathway	6/300	233/14867	0.1385	0.5814	PDGFRB, IGF1, IL7, CCNE1, TNC, EFNA1
32	path:hsa05218	Melanoma	2/300	39/14867	0.142	0.5814	PDGFRB, IGF1
33	path:hsa05214	Glioma	2/300	41/14867	0.1509	0.5952	PDGFRB, IGF1
34	path:hsa05322	Systemic lupus erythematosus	2/300	42/14867	0.1553	0.5952	C6, C9

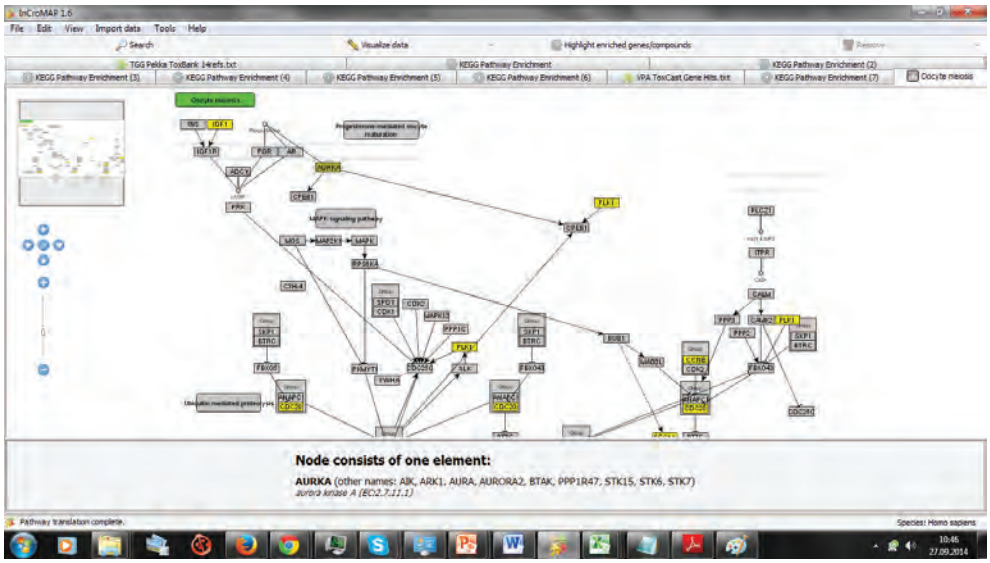

Figure 4.61 *Disturbance of the oocyte meiosis pathway following exposure to valproic acid.*

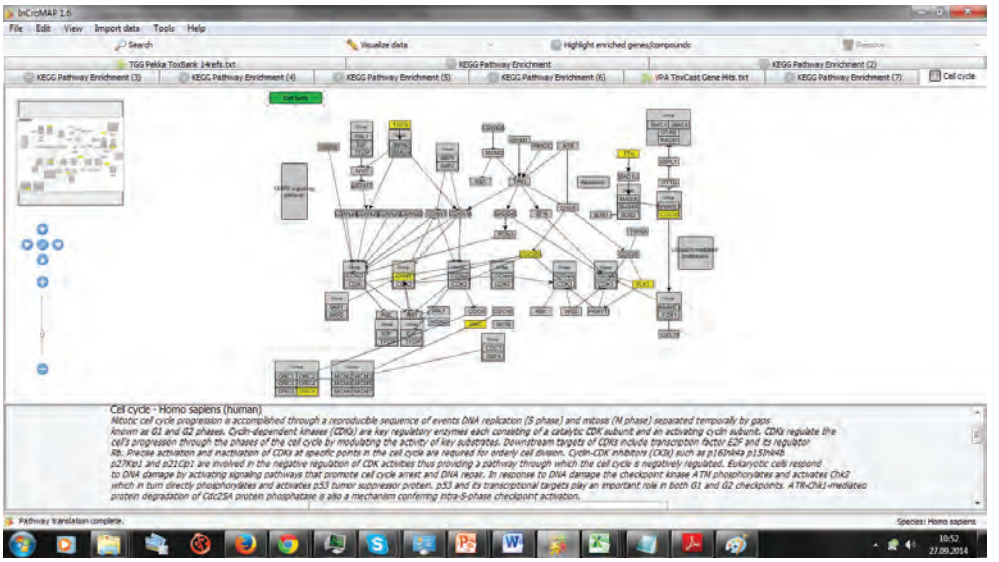

Figure 4.62 *Impact of valproic acid on the cell cycle replication process.*

The ToxCast VPA data on non-gene target assays shows activities in assays involving AHR, cell cycle, mitotic arrest, and oxidative stress and are consistent with the above TG-GATEs data. Gene target assays showed activities for p53 (managing DNA repair or apoptosis if repair not feasible), FXR (controlling bile acid synthesis from cholesterol), and CYP19A1 (controlling aromatase production converting androgen male hormones to different forms of the female sex hormone estrogen).

For multiple enrichment of TG-GATEs and gene target ToxCast assays we combined the two data sets in a multiple enrichment mapped to Kegg pathways resulting in the set of processed data shown in *Table 4.4* (note that in these enrichments we apply a filter to include *Homo Sapiens* data only). The addition of the CYP19A1 gene moves the steroid hormone synthesis pathway (*Figure 4.63*) from its previous no. 9 significance position with TGG '-omics' data (3/298 genes, p-value of 0.024) to its new no. 2 position (4/298 genes, p-value of 0.0037), inferring impact of the VPA – which may present a lipid head group like chemophore to synthesis machinery – on the conversion of cholesterol to hormonal steroids.

Table 4.4 *Combined enrichment analysis to Kegg pathways with InCroMAP using data from the TG-GATES and the ToxCast databases.*

#	ID	Name	List ratio	BG ratio	P-value	Q-value	Genes/Compounds
1	path:hsa04114	Oocyte meiosis	6/300	73/14867	2.859E-3	0.1646	CDC20, PLK1, IGF1, CCNE1, SGOL1, AURKA
2	path:hsa00140	Steroid hormone biosynthesis	4/300	33/14867	3.71E-3	0.1646	HSD17B2, CYP3A7, 1588, SULT1E1
3	path:hsa04110	Cell cycle	7/300	105/14867	4.084E-3	0.1646	CDC20, ORC6, PLK1, TGFB1, CCNE1, TTK, CDC25A
4	path:hsa00830	Retinol metabolism	4/300	36/14867	5.027E-3	0.1646	RDH16, CYP2C19, CYP4A11, CYP3A7
5	path:hsa00330	Arginine and proline metabolism	4/300	42/14867	8.464E-3	0.2218	ARG1, CPS1, OAT, PRODH2
6	path:hsa04975	Fat digestion and absorption	3/300	26/14867	0.0133	0.2902	MOGAT3, MOGAT2, MTTP
7	path:hsa05020	Prion diseases	3/300	29/14867	0.0176	0.3046	C6, HSPA5, C9
8	path:hsa00591	Linoleic acid metabolism	2/300	11/14867	0.0186	0.3046	CYP2C19, CYP3A7
9	path:hsa00380	Tryptophan metabolism	3/300	32/14867	0.0225	0.327	DDC, ACMSD, KMO
10	path:hsa00590	Arachidonic acid metabolism	3/300	34/14867	0.026	0.3409	CYP2U1, CYP2C19, CYP4A11
11	path:hsa04913	Ovarian steroidogenesis	3/300	39/14867	0.0359	0.4278	HSD17B2, IGF1, 1588
12	path:hsa00982	Drug metabolism - cytochrome P450	2/300	18/14867	0.0449	0.4295	CYP2C19, CYP3A7
13	path:hsa00062	Fatty acid elongation	2/300	19/14867	0.0492	0.4295	ELOVL7, ELOVL4
14	path:hsa01230	Biosynthesis of amino acids	3/300	45/14867	0.0494	0.4295	PKLR, ARG1, CPS1
15	path:hsa04621	NOD-like receptor signaling pathway	3/300	47/14867	0.0542	0.4295	CCL2, CARD6, TRIP6
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24	path:hsa04914	Progesterone-mediated oocyte maturation	3/300	58/14867	0.0826	0.4295	PLK1, IGF1, CDC25A
25	path:hsa04976	Bile secretion	3/300	58/14867	0.0826	0.4295	SLC10A1, 9971, SLC22A1
26	path:hsa04115	p53 signaling pathway	3/300	59/14867	0.0852	0.4295	IGF1, CCNE1, SESN3
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31	path:hsa04151	PI3K-Akt signaling pathway	6/300	233/14867	0.1385	0.5814	PDGFRB, IGF1, IL7, CCNE1, TNC, EFNA1
32	path:hsa05218	Melanoma	2/300	39/14867	0.142	0.5814	PDGFRB, IGF1
33	path:hsa05214	Glioma	2/300	41/14867	0.1509	0.5952	PDGFRB, IGF1
34	path:hsa05322	Systemic lupus erythematosus	2/300	42/14867	0.1553	0.5952	C6, C9

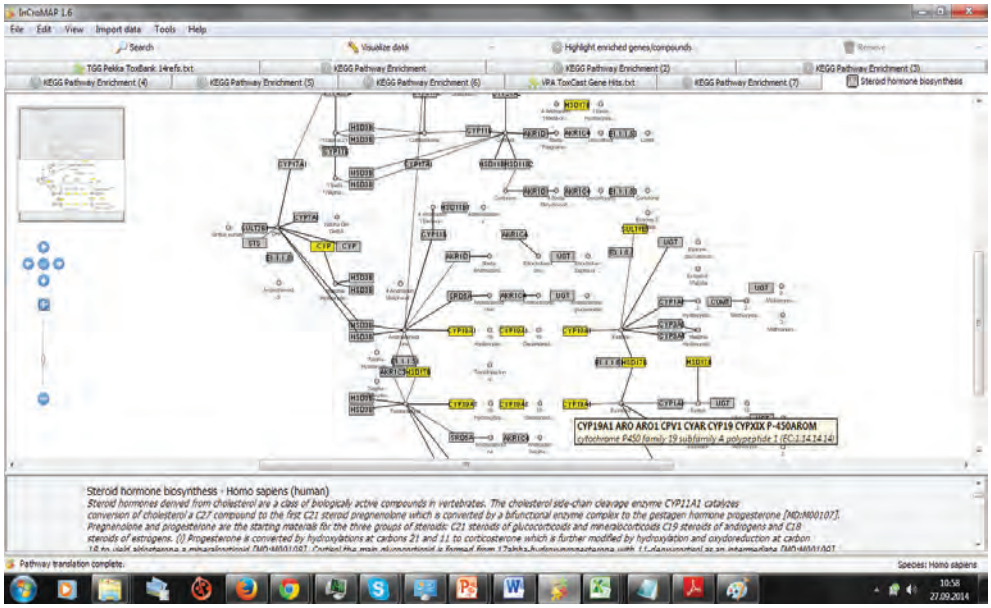

Figure 4.63 *Impact of the CYP19A1 gene on the significance position of the hormone synthesis pathway.*

Consideration of bioassay activity data of VPA in *Pubchem* (*2015*) showed a relatively small number of assays indicating activity (35) compared to in-actives (1052) and having no active with a gene-annotated *Homo Sapiens* target. However, extending the search to including chemically similar compounds we were able to find a *Homo Sapiens* target assay activity involving the salt where VPA can be considered the active ingredient. This showed activity with the succinate-semialdehyde dehydrogenase, mitochondrial (HS), which catalyses one step in the degradation of the inhibitory neurotransmitter gamma-aminobutyric acid (GABA). Inclusion of this latter gene P51649 in a multiple enrichment with the TGG data promoted the pathway for alanine, aspartate and glutamate metabolism to a significance position of 20th with a p-value = 0.076 (data not shown). Hence both data sets provide evidence supporting an influence of VPA on mitochondrial synthesis including a proposed possible drug action mechanism on GABA and a potential mechanism of toxicity through influence on the CPS1 gene which provides instructions for making the enzyme carbamoyl phosphate synthetase, whose disturbance may lead to toxic accumulation of ammonia.

Finally we considered the assay data available in Chembl which had VPA *Homo Sapiens* activities for the following targets: UDP-glucuronosyltransferase 1A4 UniProt P22310, UDP-glucuronosyltransferase 2B4 UniProt P06133, Interleukin-8 receptor B UniProt P25025, Serine/threonine protein phosphatase, 2B catalytic subunit UniProt P08209 and acetylcholinesterase 1A4 UniProt P22303. A multiple enrichment of TG-GATEs and Chembl data promoted the significance of the impact of VPA on cytokine signalling events (*Figure 4.64*) with Interleukin-8 receptor B indicated from Chembl whereas the TGG data showed influences on CCL2, PDGFRB, ACKR3, TGFB1, and IL7.

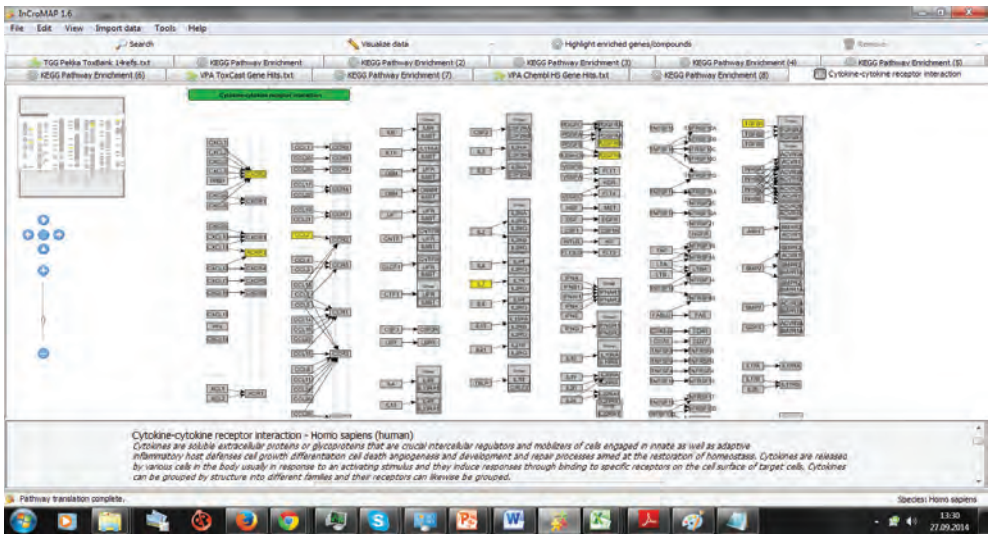

Figure 4.64 *Impact of VPA on cytokine signalling as revealed by a multiple enrichment to Kegg pathways with InCroMAP using data from the TG-GATES and the Chembl databases.*

The combined evidence provides a reasonable basis to assume that the combined mechanistic events observed from assays and '-omics' are consistent and indicate plausible toxicity pathways, some of which may contribute to steatosis. Inclusion of additional assay and '-omics' data combined with kinetic extrapolation as mapped to the steatosis AOP should provide a reasonable basis for development of risk assessment approaches based on the integrated analysis of alternative biological evidence as reported on here.

Go Analysis

We repeated the above analysis for the TGG data at medium concentration, but with an enrichment against GO ontologies rather than Kegg pathways. The top ranked ontologies were for mitotic cell cycle and xenobiotic metabolic process, and with other significant ontologies involving metabolism (including for steroids and glucose), kinase binding and aromatase activity. These results are consistent with those with enrichment from Kegg pathways, the ToxCast activities (both gene and non-gene target assays) and background knowledge.

Analysis at High Concentration

We repeated the analysis at the high concentration for TGG data with highest ranking pathways being cell cycle, retinol metabolism and p53, followed by other pathways involving metabolism, DNA replication, and TNF signalling pathways. The response is similar to the medium concentration response, with stronger signals showing that the cells are being more overwhelmed by survival efforts, indicating the medium level concentration may be a better condition for picking up lower dose mechanisms of interest in repeated dose toxicity (such as steatosis). The response is also congruent with ToxCast p53 activity but with a stronger signal at the higher concentration, whereas the steroid hormone synthesis signal is present but weaker.

We also repeated the analysis at high concentrations with Go ontologies with mitotic cell cycle and mitosis ranked highest, with other ontologies involving metabolism, proliferation and repair ranked highly. The view seems broadly similar to Kegg pathways and the medium concentration effects. Steroid metabolic processes and p53 activation are indicated as occurring significantly in line with the observed ToxCast assay activities.

4.7.4 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 4.10.3). 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.

4.7.5 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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Wrzodek, C., Eichner, J., Büchel, F., Zell, A. (2012): InCroMAP: Integrated analysis of cross-platform microarray and pathway data. *Bioinformatics*, 29: 506-508.

Project-related Publications from the ToxBank Consortium

Kohonen, P., Benfenati, E., Bower, D., Ceder, R., Crump, M., Cross, K., Grafström, R. C., Healy, L., Helma, C., Jeliazkova, N., Jeliazkov, V., Maggioni, S., Miller, S., Myatt, G., Rautenberg, M., Stacey, G., Willighagen, E., Wiseman, J., Hardy, B. (2013): The ToxBank Data Warehouse: Supporting the replacement of *in vivo* repeated dose systemic toxicity testing. *Mol. Inf.*, 32: 47–63.

Kohonen, P., Ceder, R., Smit, I., Hongisto, V., Myatt, G., Hardy, B., Spjuth, O., Grafström, R. (2014): Cancer biology, toxicology and alternative methods development go hand-in-hand. *Basic Clin. Pharmacol. Toxicol.*, 115: 50-58.

Maunz, A., Gütlein, M., Rautenberg, M., Vorgrimmler, D., Gebele, D., Helma, C. (2013): Lazar: A Modular Predictive Toxicology Framework. *Frontiers Pharmacol.,* 4: Article 38 (published 9 April 2013).

Awards

Pekka Kohonen and Roland Grafström (Karolinska Institute, Stockholm) received the 2014 Lush Science Prize

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.

Roland Grafström and Pekka Kohonen work at the Institute for Environmental Medicine, which is one of the largest research departments at the Karolinksa Institute in Stockholm, Sweden. They received the Lush Science Price for combining *in vitro* and *in silico* analyses in the context of the ToxBank project. The award was made for notable contributions to the field of predictive toxicology focussing around efforts on the application, analysis, interpretation and storage of '-omics'-derived data.

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

COACH

Sara Vinklatova, Bruno Cucinelli

4.8.1 Introduction

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

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

- Facilitate cluster-wide internal cooperation;
- Provide strategic guidance with the help of the Scientific Expert Panel (SEP);
- Prepare and distribute the **SEURAT-1** Annual Reports;
- Organise the **SEURAT-1** Annual Meetings, meetings of the SEP and workshops supporting cross-cluster activities and collaborations with external partners;
- 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 for all participants, 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 4.65*).

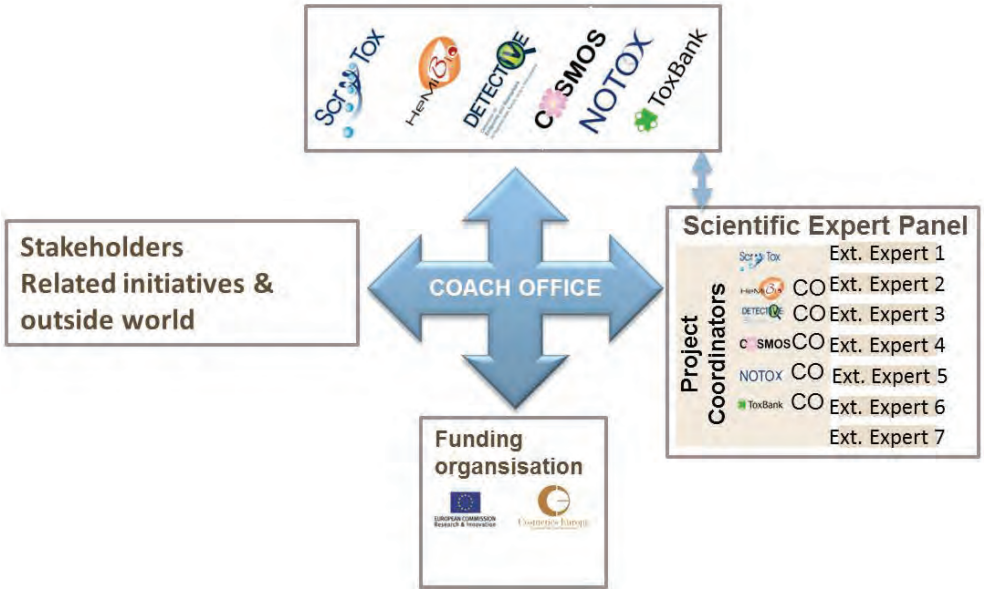

Figure 4.65 *The* ***COACH*** *Office as the central contact for cluster-level activities.*

Each of the seven projects of the **SEURAT-1** Research Initiative is governed by a contractual framework composed of contracts with the European Commission (the FP7 Grant Agreement) and the cosmetics industry association Cosmetics Europe. These contracts define 18-month work periods (reporting periods). The **SEURAT-1** Research Initiative is currently in the third 18-month reporting period, finishing in June 2015. Similar to the previous two 18-month periods, all seven projects will submit a written report in August 2015 to be evaluated by the European Commission and Cosmetics Europe. Together with external reviewers appointed for this purpose, the European Commission and Cosmetics Europe will then evaluate the individual projects progress and cluster achievements and provide recommendations for the future.

The following sections highlight some important achievements of the first, second and beginning of the third periods (illustrated in *Figure 4.66* below).

Modus operandi

- Establishment of the **Scientific Expert Panel (SEP)** and other task forces supporting the cluster activities (training task force, editorial review board, symposium organising committee...)
- Setting up of the **COACH Office** as a main contact for all cluster members and the outside world
- Successful organisation of **Annual Meetings** with a standard participation of >130 scientists
- Implementation of **e-collaboration tools** (private webspace, mailing lists, teleconferences, Webex...)

Strategy

- Definition of the **SEURAT vision** and long term research strategy
- Definition of a **strategic review** process and its implementation
- Elaboration of the regularly updated cluster **roadmap**
- Definition of eight Level-2 and three Level-3 **Case Studies** supporting SEURAT-1 Proof of Concepts
- Issuing a scientific paper on "**Recommendations for future research in the field of predictive toxicology**" aiming to advise on how SEURAT should evolve scientifically in the future

Dissemination

- Definition of a **dissemination strategy** and updating it towards the final stages of the cluster and activities in 2016
- Elaboration of a **mass media dissemination plan** in close collaboration with journalists
- Creation and update of **dissemination material** distributed worldwide via identified dissemination channels
- Organisation of dedicated **dissemination and stakeholders events** in liaison with other related initiatives
- Participation and active contribution to major **international conferences.**

Annual Reports

- Publication of four **SEURAT-1 Annual Reports** and editing of the fifth one
- Establishment of an extensive **distribution list** and an Annual Book impact **questionnaire**
- Organisation of dedicated **Book launch events** (ESOF 2012, SEURAT-1 & EPAA Stakeholders Event 2013, 9th World Congress 2014 and EUROTOX 2015)

Cross-cluster cooperation

- Launch of cross-cutting **working groups**, organisation of workshops and extension of their activities when needed
- Identification of the **cluster key deliverables** feeding the cluster roadmap allowing their close follow-up
- Creation of the **SEURAT-1 catalogue** collecting methods and tools developed in the cluster projects aiming to serve as a source of information complementary to the ToxBank database
- Supporting the execution of the **level 2 and level 3 case studies** under the SEURAT-1 Proof of Concepts

Training activities

- Creation of a cluster **training task force** representing all projects
- Harmonisation of the cluster **training strategy** and development of a training concept
- Preparation of two cluster level **summer schools**

Figure 4.66 *Main cluster-level achievements of the* ***SEURAT-1*** *Research Initiative since the launch of the initiative.*

4.8.2 Cluster-Level Coordination

As with any collaborative research initiative, the starting period for **SEURAT-1** was key to short- and long-term success. At the start of the collaboration, partners needed to establish the methods, means and common references that allowed 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 comprised 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** and beyond.

In mid-2014, **COACH** partner University of Tuebingen encouraged creation of the so-called Strategic Group. Composed of six experts selected from SEP members, the Strategic Group aimed to prepare a strategy proposal for the next research activities following **SEURAT-1** in line with the global vision and long-term strategy elaborated by the initiative. In November 2014 the paper "*SEURAT: Safety Evaluation Ultimately Replacing Animal Testing – Recommendations for future research in the field of predictive toxicology*" was issued, published in *Archives of Toxicology* (*Daston et al., 2015*) and on the **SEURAT-1** website.

At the operational level, the SEP continued to monitor the cluster-level progress made by **SEURAT-1** towards its global objectives via regular strategic reviews of **SEURAT-1**. The motivations for implementing this plan originally initiated by **COACH** 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 brainstorming exercise by **COACH**, cluster coordinators, SEP members and 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 annually 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 contributions by 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 '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.

The third strategic review was presented and discussed at the SEP meeting in Paris in May 2014 and released in June 2014. Based on a comparison of the corresponding 2012 and 2013 results with the latest version, a clear change was observed in the SWOT exercise. Further details, including the **SEURAT-1** roadmap, are given in section 4.10.1 of this Annual Report.

Collaborations with Related Initiatives

Collaborating with related research initiatives and institutions in- and outside Europe has been on top of the **COACH** action lists since the start of **SEURAT-1**. Links were established with many international actors, 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). In 2014, **COACH** also opened discussions with the MIP-DILI initiative with the objective of organising a common workshop (see section 5.3). More details about related international research programmes are summarised in section 5.2.

The second cluster training activity, called **SEURAT-1** & ESTIV joint summer school, also illustrated the role of **COACH** in supporting collaborations with related initiatives. The summer school 2014, organised by **COACH** partner ARTTIC, took place on 8-10 June 2014 in Amsterdam. Thanks to the collaboration with ESTIV, it brought together *in vitro* and *in silico* toxicologists from many different countries, representing academia, industry and regulatory bodies and the **SEURAT-1** young scientists who showcased their work within **SEURAT-1**. More details on this fruitful collaboration can be found in section 4.11.1.

The **SEURAT-1** book (Annual Report) launch events represent an excellent opportunity to deepen the relationships with important initiatives or stakeholders and gain visibility for **SEURAT-1**. In 2012, the Annual Report was launched at EuroScience Open Forum (ESOF) 2012; in 2013 during the **SEURAT-1** & EPAA Stakeholders Event; and in 2014 during the 9th World Congress on Alternatives and Animal Use in the Life Sciences (WC9). The launch of the 5th Annual Report will build on this tradition and will be organised at the EUROTOX 2015 conference in September 2015 in Porto, Portugal. Similarly to the WC9, **COACH** will coordinate the **SEURAT-1** corner, hosted at the Joint Research Centre booth, distribute **SEURAT-1** dissemination material (leaflets, posters, USB sticks, all volumes of the Annual Reports, etc.) and arrange associated activities such as interviews, short scientific sessions, demonstrations, etc.

4.8.3 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** set up e-collaboration tools at the outset of the initiative, and these have been used intensively since their creation. Besides dedicated mailing lists, **COACH** provides a collaborative web platform, operated by partner ARTTIC, which facilitates the sharing of information and remote collaboration. The **SEURAT-1** private workspace is accessible for registered users who are involved in the cluster projects, the European Commission and some experts of Cosmetics Europe who signed a special Non-Disclosure Agreement.

The **SEURAT-1** Annual Meetings are the main face-to-face events for gathering the cluster participants. The first two Annual Meetings (March 2011 and February 2012) were organised with a similar concept: (i) a plenary session involving a series of keynote speeches about important issues in alternative human safety testing international research, including progress made by the cluster projects; (ii) parallel working groups focusing on specific cross-cluster topics; and (iii) a panel discussion drawing conclusions from the discussions and providing a common view on future work orientations and priorities of the research initiative. The third 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. The fifth Annual Meeting (January 2015) also had the objective of stimulating discussions on how to successfully conclude its activities by the end of the year. This last meeting in January 2015 showcased the impressive outcomes of the research project and initial results from the **SEURAT-1** proof-of-concept (PoC) case studies (aiming to be finalised by the end of 2015; see sections 3.3–3.5). A non-exhaustive list of the tools and methods for mechanism-based toxicology developed in the **SEURAT-1** projects was presented in a dedicated session and it was agreed that all methods will be collected as a common outcome in a catalogue to be made available at the end of 2015 (this is supposed to be the '**SEURAT-1** Tools & Methods Catalogue', see section 4.10.2). It has become tradition that three **SEURAT-1** young scientists are granted with 'Excellent Poster Awards' at the Annual Meeting. The winners of the 2015 award were: Juan Diaz (NOTOX), Adil El Taghdouini (*HeMiBio*) and Sophie Teng (COSMOS) (extended abstracts are given in section 4.9.3).

Another important element of fostering collaborations between the scientists in the different research projects is the organisation and maintenance of cross-cluster working groups. A detailed overview of these working groups is given in section 4.10.3 and activity reports are presented in sections 4.10.4–4.10.9. Besides actively preparing workshops, **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, easing communication and collaboration among the working group members. The **COACH** partner Joint Research Centre assisted in the coordination of the Level 2 and Level 3 case studies for the **SEURAT-1** proof-of-concept exercises. In 2014, two workshops were organised in Ispra, setting the scene for the Level 3 read-across case study (29-30 April) and the *ab initio* case study (9-10 October). At the read-across workshop, relevant experts, also external to **SEURAT-1**, were invited to ensure a robust and well-informed basis for the project. The outcome of the workshop was also published in a review paper in *Environmental Health Perspectives* (*Berggren et al, 2015*). Both these case studies will be presented at the final **SEURAT-1** symposium in December 2015. In addition they will also feed into the ECHA scientific topical workshop on New Approach Methodologies in Regulatory Science, to be held on 19-20 April 2016 in Helsinki, Finland.

Internal training encourages exchange between younger scientists and facilitating the knowledge transfer developed within **SEURAT-1**. The training concept was homogenised by a special **SEURAT-1** training task force composed of representatives from each of the projects. In 2014, **COACH** organised the second cluster-level summer school (8 - 10 June) in close collaboration with the ESTIV2014 conference. The **SEURAT-1** part of the summer school covered mostly practical sessions (hands-on computer exercises, soft skills sessions, discussions and workshops), while the ESTIV programme offered the participants scientific conferences and a career session. Section 4.11.1 provides further details on the training activities.

4.8.4 Information Dissemination

The **COACH** dissemination activities evolve together with **SEURAT-1**, as each phase of its duration requires specific attention. Since 2011 the activities developed from creating the **SEURAT-1** visual identity as a new player in the field of alternative testing and presenting the objectives of the Research Initiative to the entire scientific world, to showcasing the first achievements to the key stakeholders, the general public and other target groups via a number of channels. The following activities and tools were developed to support the dissemination as efficiently as possible:

- A consistent visual identity for **SEURAT-1** (logo, colours, layout of printed and electronic dissemination material, website appearance, etc.) has been developed at the outset of the initiative in collaboration with a professional design company;

- A variety of information dissemination support materials has been created and distributed, including: a first version of the leaflet; second version of the leaflet containing an embedded USB stick; **SEURAT-1**, **COACH** and cluster roadmap posters; a 'who's who' booklet, which is distributed at each Annual Meeting (also available online); a standard PowerPoint presentation presenting the initiative and showcasing the initial results; unique roll-up banners illustrating the cluster composition and the conceptual framework, which were used during the **SEURAT-1** & ESTIV joint summer school and at the **SEURAT-1** corner at the 9th World Congress on Alternatives and Animal Use in the Life Sciences in Prague, Czech Republic, in August 2014;

- The public website (www.seurat-1.eu) is kept up to date. It presents the Research Initiative and its background and aims, the cluster projects and the involved partner organisations, and promotes the research activities in the field of human safety assessment, in particular regarding alternatives to *in vivo* repeated dose systemic toxicity testing. Dedicated, regularly updated pages

present related events, links, publications, job announcements, etc. Recently it was enriched by adding a section called Press Corner aiming to help journalists quickly and easily find key **SEURAT-1** information of interest to the general public;

➠ The preparation of the **SEURAT-1** Annual Reports was coordinated by **COACH** partner University of Tuebingen, 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 the validation of the book's contents. The University of Tuebingen collected, reviewed and edited the contributions while ARTTIC took care of the book layout in collaboration with an appointed professional designer. Further to this well-established collaboration, the following Annual Reports were published and distributed in more than 1000 copies each:

- First Annual Report (*Schwarz & Gocht, 2011*) published in September 2011;
- Second Annual Report (*Gocht & Schwarz, 2012*) published in July 2012 and launched at the Euroscience Open Forum (ESOF) in July 2012;
- Third Annual Report (*Gocht & Schwarz, 2013*) published in July 2013 and launched at the **SEURAT-1** & EPAA Stakeholders Event in September 2013;
- Fourth Annual Report (*Gocht & Schwarz, 2014*) published in August 2014 and launched at the 9th World Congress in August 2014;
- This fifth Annual Report, to be launched at the 51st Congress of the European Societies of Toxicology in September 2015 (EUROTOX 2015).

The Annual Reports were printed and distributed to individuals by post and at relevant conferences (further details on the event are given in section 4.11.3). The electronic versions of the Annual Reports are also made available for download from the **SEURAT-1** public website and distributed using USB sticks, thereby 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. It is regularly updated each year before the Annual Report distribution;

➠ The **COACH** partners are aware of the importance of promoting the objectives, approach and progress of **SEURAT-1** is at international conferences

and workshops. Participation in such events with the aim of presenting the progress and first achievements has strongly contributed to increasing the visibility of the **SEURAT-1** Research Initiative in the scientific community. The events where **SEURAT-1** was present are listed in section 4.11.3;

➠ The excellent visibility of **SEURAT-1** and its recognition as the major European research initiative in the field of alternative human safety testing methods is the fruit of a dissemination plan prepared as a project internal working document by **COACH** in 2011. It defined the dissemination objectives and the appropriate means required to reach the targets, leading to a number of actions. The research community should of course also remain an important target for information dissemination, exchanges and collaboration, but, as already reported in the last volume of this book, a need occurred 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 in 2013. Aiming to define dissemination objectives, means and channels to better target the stakeholder groups, and to establish a plan of appropriate concrete dissemination actions, this dissemination strategy is considered as a living document to be reviewed in each SEP meeting;

➠ Associated with the above-mentioned dissemination strategy, a plan for communication in mass media was developed in 2014. The creation of the plan was initiated by the **COACH** partner ARTTIC and supported by the newly set Editorial Review Board containing representatives from the SEP, European Commission and Cosmetics Europe. Its objective is to reach out to public journals, radio, television and other media outlets to spread information about the **SEURAT-1** results.

As the **SEURAT-1** Research Initiative moves closer to the termination of the research projects, dissemination of the scientific results becomes more and more important. The dissemination strategy and the mass media communication plan are now the key references for the **SEURAT-1** dissemination activities and will also serve as the basis for identifying tasks to be performed in 2016 by **COACH**, i.e. to disseminate the **SEURAT-1** results and hand over the cluster results to the future research initiative(s) in the most efficient manner.

4.8.5 Next Steps

The **COACH** action list currently includes the following work topics:

Dissemination to stakeholders and general public; close collaboration with related and future

initiatives: As detailed above, **COACH** is now more than ever concentrating its efforts on the organisation of **SEURAT-1** events with international participation, mainly via the **SEURAT-1** symposium; and on the active contribution at well-known conferences such as SOT 2015 or EUROTOX 2015. All events will be underpinned by well-coordinated, wide-ranging communication with target groups as well as mass media and the general public, as defined by the **SEURAT-1** Editorial Review Board in the dissemination strategy. All dissemination material created so far will also support these actions to increase **SEURAT-1** visibility, and the entire **SEURAT-1** Research Initiative will be mobilised to spread the generated information via their individual networks.

Organisation of the **SEURAT-1** Symposium entitled *Painting the future animal-free safety assessment of chemical substances: Achievements of **SEURAT-1***. The 6th and final Annual Meeting, organised in the form of a public symposium, will take place on 4 December 2015 in Brussels. It will be of the highest interest for policy makers, regulators, industry, the scientific community and animal welfare groups, but also for the general public and the press. Presentations on progress achieved in animal-free testing strategies by **SEURAT-1** will first showcase the **SEURAT-1** success stories in a practical and accessible manner. An exhibition with demonstrations organised at dedicated information booths will then allow for deeper discussions. The attendees will learn how the extensive research efforts during the last 5 years can be translated into solutions for safety assessment ultimately replacing animal testing. Other related on-going and future initiatives from the EU and US will be invited to showcase their progress in the field of alternative testing strategies and thus stimulate exchange and networking. More information and instructions for registering for the event are available on www.seurat-1.eu.

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 the long research effort required to develop alternative solutions for human safety assessment with a view to replacing animal testing. **SEURAT-1** research projects are approaching their end in December 2015, and the partners and stakeholders are becoming very active, especially in preparing the dissemination of the individual projects' and the clusters' final results. **COACH** will continue to stimulate and coordinate these tasks with the aim of handing over the **SEURAT-1** knowledge to the future research initiative(s) in the most efficient way and thus approaching the achievement of the SEURAT long-term goals.

Priority work topics for the fifth year will also address the finalisation of the achievements made in the past periods, i.e., report on the final results of the proof-of-concept case studies and of the cross-cluster Working Groups, preparation of the next training activities and even closer collaboration with related research initiatives and organisations.

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4.9 Project and Cluster Activities

4.9.1 Project Meetings

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***SCR&Tox*:** 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 fourth year: The 42 months progress report meeting took place on 1–2 July 2014 in Oslo and the third *HeMiBio* Annual Consortium Meeting on 14–15 January 2015 in Chur, Switzerland. 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. 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 *in vitro* biokinetics was held just before the Annual Meeting on 13 January 2015 in Chur, Switzerland (a summary is given in chapter 4.11.1). Finally, the three-monthly web-conference has concentrated on the biological aspects of the project and in particular on cell engineering progress.

DETECTIVE: The fourth DETECTIVE General Assembly took place on 3–4 February in Barcelona. The DETECTIVE advisory board attended the meeting. A significant progress across the different work packages had been demonstrated. It has been concluded that the use of repeated dosing and recovery periods represented a significant advantage to maximise the rationale for the time component for repeated dose translation. Additionally, DETECTIVE was leading a way providing consortia level data and working with additional partners. Advanced approaches for biomarker integration were translated into novel reporter assays. Three case studies have been finally concluded and initiated (further information about the case studies

can be found in sections 3.3.1, 3.4.3 and 3.4.4). Several meetings were organised last year to consolidate the biomarker strategy across DETECTIVE partners and the target organs heart, liver and kidney. The DETECTIVE advisory board was strongly involved in the biomarker strategy development and guiding. Additionally, DETECTIVE partners have contributed to the **SEURAT-1** and ESTIV Joint Summer School on 8–10 June in Egmond aan Zee. DETECTIVE achievements were presented at the 9th World Congress on Alternatives and Animal Use in the Life Sciences (WC) on 24–28 August in Prague.

COSMOS: The COSMOS Fourth Annual General Meeting took place in Barcelona, Spain on 3-4 February 2014, before COSMOS delegates joining the fourth **SEURAT-1** Annual Meeting on 5-6 February 2014. The status of the work 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 physiologically-based pharmacokinetic (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, as well as the contributions to the COSMOS symposium session at SOT 2014, were planned. At the **SEURAT-1** Meeting 2014, COSMOS contributed to the poster session, with two poster winning poster awards, COSMOS members contributed to several discussion sessions, including the case study discussions and the Biokinetics Working Group co-lead by COSMOS.

An informal COSMOS meeting took place on Monday 16 June 2014 at the Mario Negri Institute in Milan, Italy before the start of the QSAR2014 Workshop. In addition, a work package 2 discussion meeting on the Threshold of Toxicological Concern approach with external guests was held after the QSAR conference on Friday 20 June 2014.

The six-monthly COSMOS General Assembly meeting was hosted by COSMOS partner Molecular Networks on 24-25 September 2014 in Erlangen, Germany. COSMOS working group and work package discussions took place in addition to the review and planning of the next steps of the project. In addition, a mini KNIME workshop was held with demonstrations of workflows developed within COSMOS. Importantly, the planned major outcomes at the end of the project in terms of available models and associated documentation, high profile publications, dissemination as well as legacy and maintenance of COSMOS tools were laid out.

On 25 November 2014, a workshop was hosted by **COSMOS** partner KNIME in Zurich, Switzerland, to discuss remaining steps for the public launch of the models and workflows in

the COSMOS KNIME WebPortal, including a standardisation of the layout and content of the workflow descriptions, reports as well as related documentation and guidance.

At the end of the fourth project year, the COSMOS fifth Annual General Meeting took place in Barcelona, Spain on 19-20 January 2015, before COSMOS delegates joined the fifth **SEURAT-1** Annual Meeting on 21-22 January 2015 in Barcelona. In addition to the plenary meeting with review of the project status, update of the final outcome, legacy road-map and sustainability plan, group discussions were held to discuss details for example of the envisaged Cosmetics Space tool and further improve the KNIME workflow output. COSMOS contributed to the **SEURAT-1** meeting with the yearly update on the project, presentations of COSMOS Level 2 and Level 3 case studies and Tools and Methods, as well as involvement in the different case study discussion sessions. 12 COSMOS posters contributed to the **SEURAT-1** project achievements and case study poster sessions, two posters winning poster awards.

In addition to these meetings, COSMOS partners and work packages held many additional informal meetings or teleconferences in small groups to discuss specific questions for the on-going work. Furthermore, the ILSI-EU COSMOS TTC Expert Groups met frequently. The 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 Threshold of Toxicological Concern approach to cosmetics-related substances.

NOTOX: 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 on 10 June 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.

NOTOX partner Insphero organised a workshop entitled '2D and 3D human liver cell bioreactors for long-term repeated-dose toxicity testing as a basis for computer-aided prediction' on 16 May 2014 in Heidelberg. The NOTOX coordinator Elmar Heinzle hold a lecture about '3D Spheroid Cultures of Human Liver Cells for Long-term Repeated-dose Toxicity Testing' on this occasion.

4.9.2 Cluster Meeting of the SEURAT-1 Research Initiative

Elisabet Berggren

The **SEURAT-1** Research Initiative assembled for its fifth annual meeting in Barcelona on 21-22 January 2015 with the purpose to report major achievements and progress since the last annual meeting and discuss how to successfully conclude its activities by the end of the year.

The research projects presented impressive results (also highlighted in sections 4.2-4.6 of this report). DETECTIVE reported identification of novel biomarkers for kidney, liver and heart. They also showed that differentiated human-skin derived cells can acquire hepatic properties, e.g. they demonstrate steatotic functions when exposed to a chemical known to cause fatty liver, and therefore they are suitable to use for *in vitro* hepatotoxicity testing. All cell systems tested within DETECTIVE were found to have a toxicological memory in their genetic profile after exposure to chemical stressors. NOTOX demonstrated the results of a multi-scale model for steatosis prediction including repeated dose 3D *in vitro* models, using HepaRG, in combination with computational biokinetic modelling mimicking human exposure. The COSMOS DB with a record of more than 80.000 cosmetic relevant substances is now publicly available and is further complimented with a TTC (Threshold for Toxicological Concerns) database, 'COSMOS space' with representations of the chemical space of cosmetic ingredients and easy-to-use biokinetic models in the open source software KNIME. COSMOS also developed several profilers that can be applied to characterise chemicals, for example binding potential to specific proteins or skin penetration coefficients. *SCR&Tox* have finalised protocols for differentiations of human pluripotent stem cells into keratinocytes, neurones, cardiomyocytes and hepatocytes, and in addition established technologies for large-scale production and banking. *HeMiBio* presented results from a unique model predicting fibrosis composed of 3D co-cultured liver cell lines in a flow over bioreactor with implemented electronic sensors for detection of read-outs. ToxBank reported good progress in collecting resulting data from the **SEURAT-1** projects as well as the proof-of-concept case studies.

Results from the eight **SEURAT-1** Level 2 proof-of-concept (PoC) case studies were presented and discussed (see also section 3.4). These case studies are tailor-made to predict specific types of toxicity based on **SEURAT-1** methods. They are based on Adverse Outcome Pathway reasoning to target specific adverse effects with relevance to human disease due to repeated dose exposure. Final conclusions are still to be summarised and one of the more interesting results will be to evaluate how the different case studies managed to meet the challenge in predicting long-term effects based on *in vitro* exposure scenarios.

There are three **SEURAT-1** Level 3 PoC case studies, for which chemicals safety assessment are carried out based on: (i) an *ab initio* approach (no animal data available on the chemical itself or similar chemicals); (ii) read-across from data-rich source substances to the substance to be assessed using strengthening arguments from alternative data; and (iii) accepting a

Threshold of Toxicological Concern (TTC) to be good enough based on improved substance characterisation using computational models. All three case studies apply a conceptual framework for chemicals safety assessment developed within **SEURAT-1**. The level 3 case studies are planned to be finalised by the end of 2015 and will be as far as possible based on data from **SEURAT-1** methods. If the case studies not manage to fulfill regulatory acceptance they will make the basis to better specify where further development and research is needed.

It was agreed that the tools and methods from all the **SEURAT-1** projects will be collected as a common outcome in a catalogue to be made available at the end of 2015. The DB-ALM (the European Commission's database on alternative methods) will be hosting the catalogue and the OECD accepted DB-ALM format to report alternative methods applied. Methods reported in DB-ALM will be linked to the ToxBank records containing more detailed and in certain cases confidential information and raw data. The catalogue will be an overview of the methods and of complementary utility to the information collected in ToxBank.

The annual meeting was finalised by a plenary discussion with the **SEURAT-1** Scientific Expert Panel. The panellists expressed that they were impressed of the methods developed within **SEURAT-1** presented at the meeting, but even more they were impressed in the way they were presented. The cluster is exceptionally multidisciplinary and from the beginning it had not been easy to communicate in this community. However, the partners had developed a common language. The biokinetic modelling that had been identified as a major knowledge gap from the beginning, was now commonly used and had finally become an integrated part of the projects. It was also recognised that the toxicological knowledge had grown and was used in method development and in interpretation of results. The Adverse Outcome Pathway thinking had generally been embraced and had proven to work as a backbone to organise the large amount and variety of data generated and collected within **SEURAT-1**. The conceptual framework for safety assessment based on alternative methods, toxicological knowledge and existing data, developed within the **SEURAT-1** was already considered a success. It was recognised that especially for the case studies there were still a lot of hard work to be accomplished in the last year of **SEURAT-1**, but everyone was looking forward to the final results and recognised the importance to bring the **SEURAT-1** experience into future initiatives aiming on the development of an efficient and reliable animal-free chemicals safety assessment.

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 culture conditions of hepatic stellate cells with the aim to use them in the *HeMiBio* liver bioreactor, a multi-scale whole body model for the description of the molecular mechanisms of valproic acid, and the adaptation of a PBPK model to predict tissue concentrations of valproic acid and methotrexate. 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.

4.9.3 Young Scientist Poster Award

In total, 36 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.

4.9.3.1 Development of Culture Conditions to Revert the Activated Phenotype of Cultured Human Hepatic Stellate Cells

Adil El Taghdouini, Mustapha Najimi, Pau Sancho-Bru, Etienne Sokal, Leo A. van Grunsven

Introduction

Liver fibrosis, or scarring of the liver, is a chronic wound-healing response characterised by excessive formation and accumulation of matrix proteins (*Bataller & Brenner, 2005*). Hepatic stellate cells (HSCs) have been identified as key players in fibrosis (*Mederacke et al., 2013*). In the healthy liver, quiescent HSCs (qHSCs) represent approximately 8-15% of the resident cells and are strictly located in the space of Disse, a virtual space between the hepatocytes and the liver sinusoidal endothelial cells. They are characterised by abundant cytoplasmic lipid droplets containing approximately 80% of our total vitamin A reserve, a low contractility and proliferation rate and a very well balanced extracellular matrix (ECM) homeostasis. A main event during fibrogenesis is the activation of HSCs, during which they transdifferentiate from cells with a quiescent phenotype into cells with a fibrogenic myofibroblast-like phenotype (*Friedman, 2008*). *HeMiBio* proposes to generate a liver-simulating device that could serve to evaluate the pro-fibrogenic properties of chemicals. In this specific set-up, the identification of potential pro-fibrotic compounds will mainly be based on their ability to induce HSC activation. However, human qHSCs for such screenings are not readily available due to donor shortage and the fact that isolated cell populations are culture expanded, during which they quickly acquire an activated phenotype. To address this problem, we developed a selfmade medium, called the $M3^+$ medium, that induces a quiescent-like phenotype in culture expanded, fully activated HSCs (aHSCs).

Approach

Non-parenchymal cell fractions (NPFs) are obtained after a two-step collagenase perfusion of the left liver segment (*Najimi et al., 2007*), followed by filtration and low speed centrifugation steps to discard parenchymal cells (*Figure 4.67A*). Enriched populations of qHSCs were obtained from cryopreserved NPFs by performing a density-gradient centrifugation, using 8%

Nycodenz® (*Guimarães et al., 2010*). Upon seeding of the HSCs, the cells activate (partly due to the hard plastic surface (*Olsen et al., 2011*) and become strongly proliferative, allowing us to expand the cells and further increase the purity (>98% after splitting; *Figure 4.67B*). For reversion to quiescence-like, aHSCs were incubated with M3⁺ for 5 days and reverted HSCs (rHSCs) were characterised at the phenotypic, functional and transcriptomic level (*Figure 4.67C*).

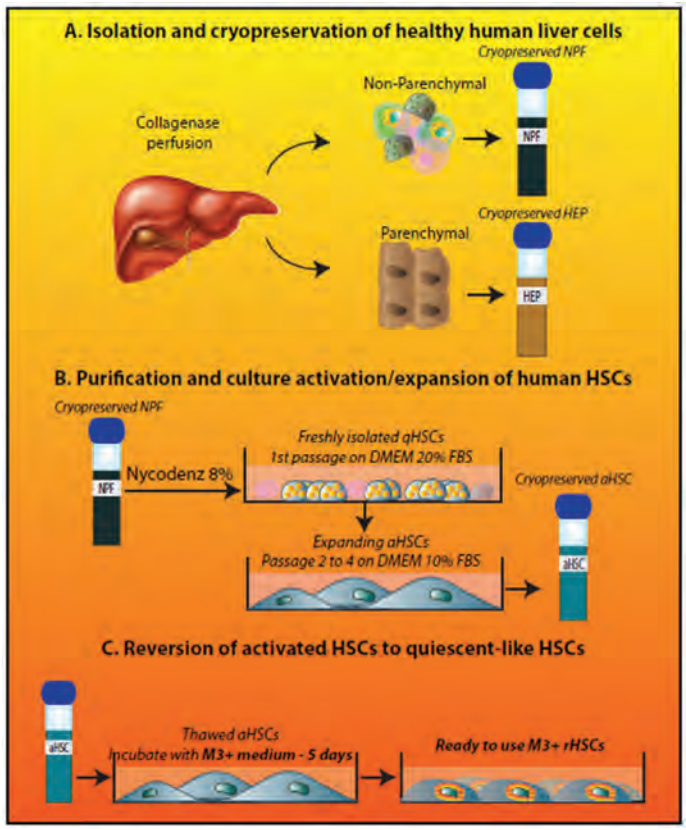

Figure 4.67 *Illustration of the followed method to isolate, activate and reverse human hepatic stellate cells.*

Results and Discussion

HSCs reverted by the M3⁺ medium display a quiescent-like phenotype, characterised by the presence of intra-cytoplasmic lipid droplets (*Figure 4.68A*). We further assessed whether rHSCs have the molecular machinery both to metabolise and store vitamin A, a functional hallmark of qHSCs, by measuring the auto-fluorescence of retinyl esters at a wavelength of 328 nm (UV-light) by fluorescence-activated cell sorting (FACS). We find that ~70% of

the rHSCs display high retinyl-ester content, while virtually all aHSCs are negative (*Figure 4.68B*). We further assessed the transcript levels of the classical activation and pro-fibrogenic markers *ACTA2*, *COL1A1* and *LOX*. These genes encode for smooth muscle actin, conferring HSCs with the potential to contract, and the main constituent and enzymatic stabiliser of fibrotic scar tissue, respectively.

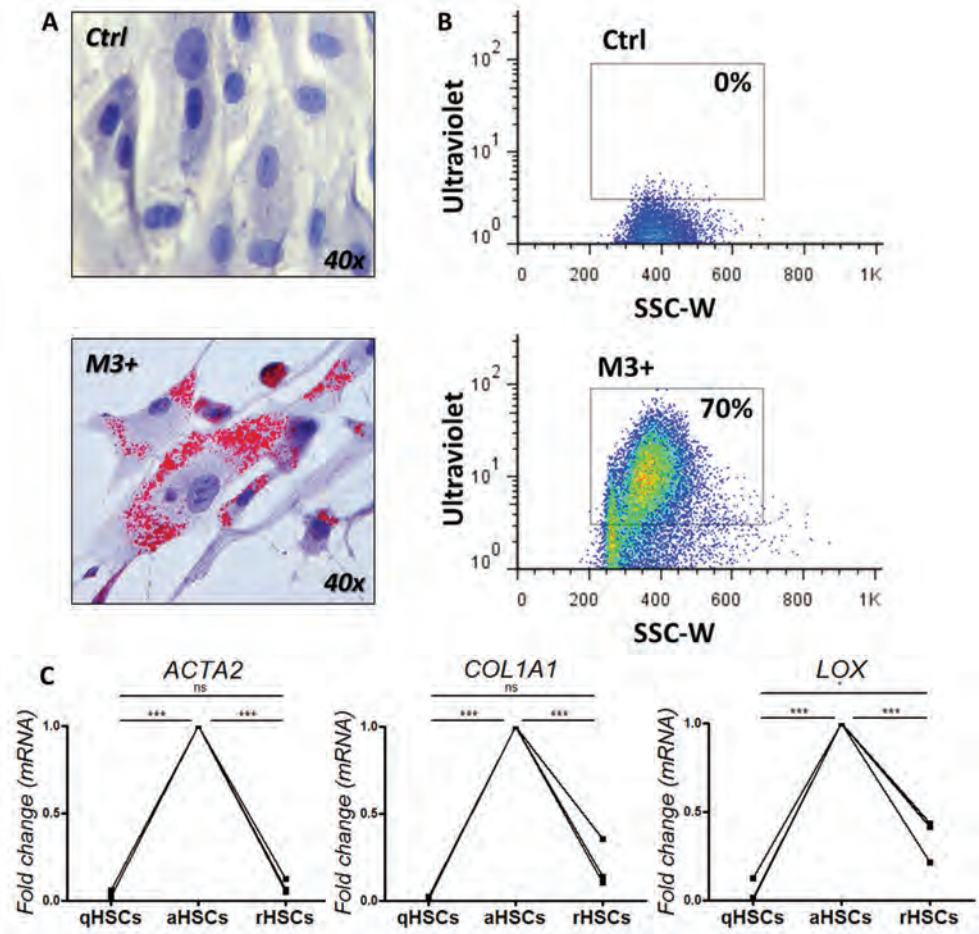

Figure 4.68 *A: Oil Red O staining images of aHSCs and rHSCs. B: FACS-detection of all-trans retinyl esters in UV-excited aHSCs and rHSCs. C: Relative mRNA expression levels of ACTA2, COL1A1 and LOX in qHSCs, aHSCs and rHSCs.*

We find that M3+ negatively regulates the expression of these genes to reach levels of expression similar to those measured in freshly isolated, uncultured qHSCs (*Figure 4.68C*). At the functional level, an EdU-incorporation and in situ zymography assay revealed that

rHSCs have a reduced proliferation and ECM degrading potential (data not shown). Together, these observations are particularly interesting since the rHSCs are still subject to the strong activating stimuli of the hard plastic surface, illustrating the potency of $M3^+$ in reverting the activated phenotype and prompting us to further uncover the events underlying this reversal in phenotype. Gene expression profiling (Affymetrix HG-U219 genechips) of uncultured qHSCs and *in vitro* aHSCs and rHSCs allowed us to identify ~400 genes that are differentially regulated after reversion by $M3^+$. Around 75% of these genes were found to have an inversely correlating expression profile between activation and reversion of HSCs, i.e. up-regulation during activation, donwregulation upon reversion and vice versa (*Figure 4.69*). This set of genes included many well-known pro-fibrogenic genes and enriched for different KEGG-pathways of major importance in aHSCs, i.e. "Regulation of actin cytoskeleton" (p-value: 9.47×10^{-5}) and "ECM receptor interaction" (2.13×10^{-3}).

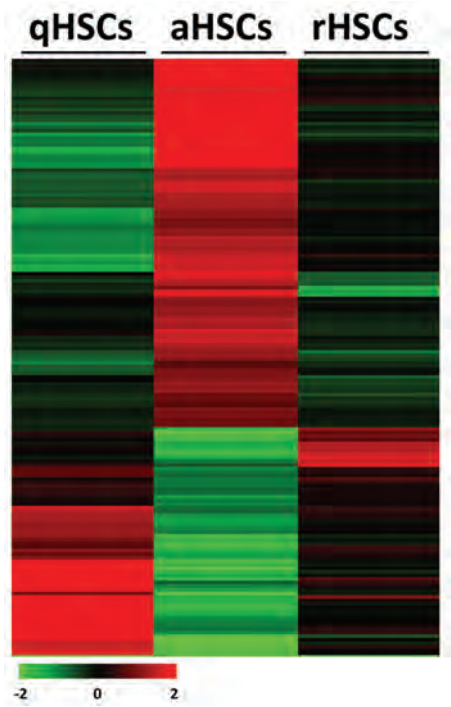

Figure 4.69 *Heatmap of relative expression levels of genes classified based on expression patterns in qHSCs, aHSCs and rHSCs.*

Conclusions

We developed the $M3^+$ medium that induces a quiescent-like phenotype in culture aHSCs. Such $M3^+$ rHSCs, display quiescent characteristics and are used in a 3D co-culture setup with

HepaRG cells in the level 2 case study carried out by *Hemibio* 'Investigation of the fibrotic response induced by methotrexate and acetaminophen in the *HeMiBio* liver bioreactor'.

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4.9.3.2 Coupled Modeling of PBPK and Toxic Mechanisms of Action of Valproic Acid in Liver

Joachim Bucher, Juan Diaz, Klaus Mauch, Lothar Terfloth

Introduction

Cellular metabolic and toxicity-related mechanism of action models coupled to pharmacokinetic or physiologically-based pharmacokinetic (PBPK) models provide insight into the relation of route and dose of administration to local effective toxic concentrations. The goal of this work is to provide a multi-scale whole body model describing the molecular mechanisms of valproic acid (VPA).

Approach

Here, a PBPK model of valproic acid is combined with a model of the hepatic VPA metabolism, based on a set of coupled ordinary differential equations, as well as toxic mechanisms/mode of action (MoA) steps which attenuate cell viability due to toxic metabolites and disturbance of lipid metabolism.

The structured tissue and vascular flow model reflects heterogeneity and zonation in liver lobules with respect to clearance and toxic response. A simplified liver tubular flow model coupled to a PBPK model is shown in *Figure 4.70*. Coupling of PBPK models with structured liver lobule and cellular network models was reported previously (*Diaz Ochoa et al., 2012; Niklas et al., 2013*).

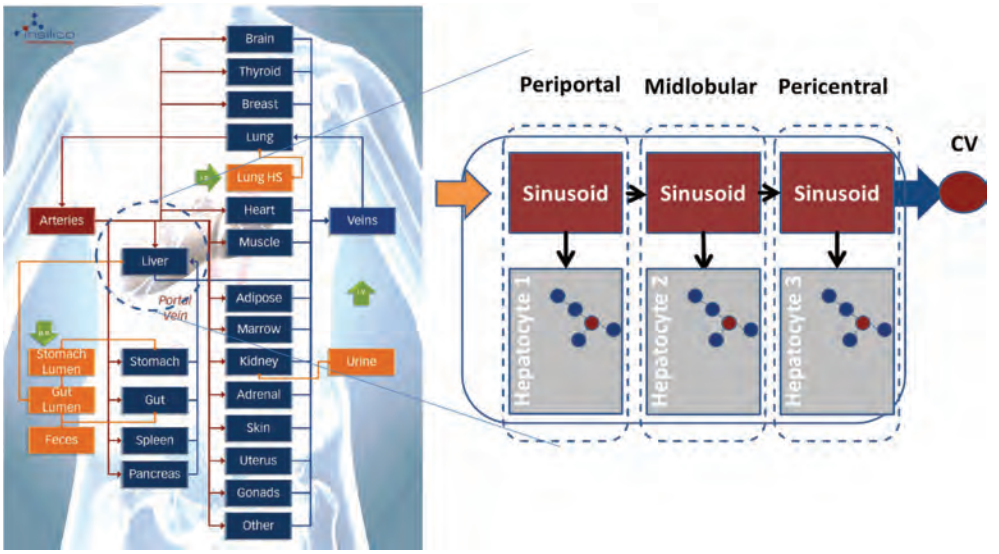

Figure 4.70 *Schematic representation of a 1D liver model containing the transport of substances between different sinusoidal sections, the transport from the sinusoid to the hepatocytes, and the molecular network in the hepatocyte. At the end of the sinusoid the substance is transported to the venous compartment through the central vein (CV).*

Results

The toxic MoA through VPA exposure comprises the long-term up-regulation of fatty acid (FA) synthesis, down-regulation of fatty-acid oxidation, direct short-term competitive inhibition of beta-oxidation by VPA metabolites, and oxidative damage by reactive VPA metabolites (*Figure 4.71A*). Coupling of this VPA toxic MoA model with Insilico's human PBPK model enables *in-vitro*-to-*in-vivo* extrapolation (*Figure 4.71B*).

The coupled PBPK-VPA-MoA-model was pre-set with a-priori-parameters, some from literature (*Kiang et al., 2006; Argikar & Remmel, 2009*), to fulfil the following demands: (i) the coupled PBPK-VPA-MOA-model was adapted to reflect *in vivo* plasma concentration profiles (*Mihaly et al., 1979*); (ii) the PBPK-VPA-MoA-model was extended to reflect potential VPA-triggered dynamic response in key-players of FA synthesis and oxidation (*Figure 4.72*). Experimental metabolite data as well as measured transcriptomic and protein data contributed by NOTOX and ToxBank will serve for model verification.

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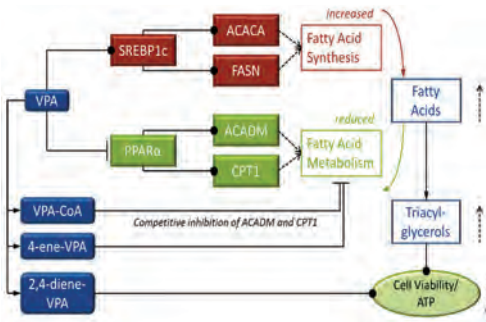

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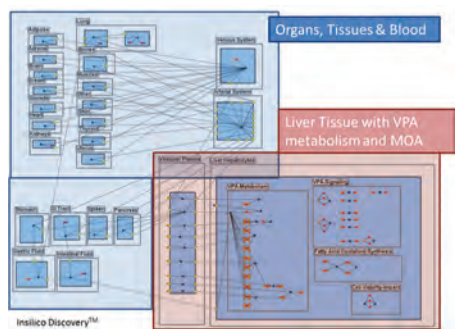
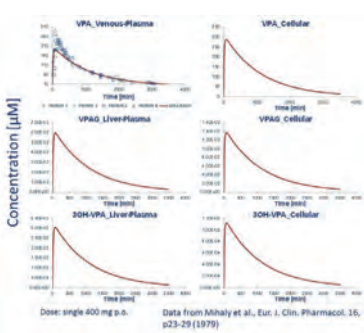


Figure 4.71 *A) The cellular MoA (Mode of Action) model captures the impact of VPA (Valproic Acid) on fatty acid and triglyceride turnover in hepatocytes. VPA modulates genes involved in FA (Fatty Acid) synthesis and oxidation via nuclear receptors SREBP and PPARalpha, respectively (Wang et al., 2012). Further, VPA metabolites directly inhibit enzymes involved in the FA oxidation leading to accumulation of triglycerides and long-term cell viability reduction. The short-term toxic effect is oxidative damage by reactive VPA metabolites. (B) The VPA-MOA-model integrated into the liver compartment of Insilico's PBPK model. Previous work demonstrated how to couple signalling and metabolic kinetic networks (Ryll et al., 2014).*

A



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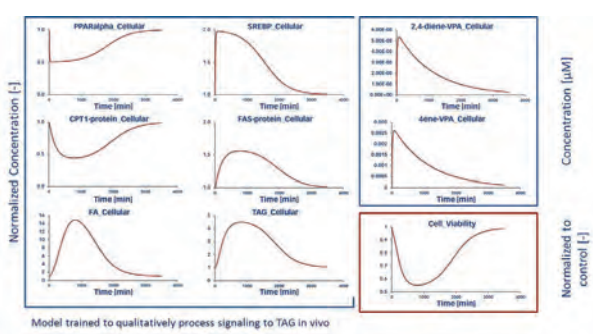

Figure 4.72 *Simulation profiles of VPA PK and MoA by the integrated VPA-MoA-PBPK-model. (A) The coupled model was a-priori parameterised and calibrated to reflect human plasma concentration profiles (Mihaly et al., 1979). Depicted are profiles of the parent compound, the UGT-glucuronidation metabolite (VPAG) and the CYP-oxidation metabolite 3OH-VPA,*

in the plasma and cellular compartment, respectively. (B) The model was further calibrated with pre-set parameters to describe the triggering of VPA-related response in key-players of FA synthesis and metabolism. VPA-response signaling could be described qualitatively in the nuclear receptors PPARalpha and SREBP, the enzymes CPT1 and FAS, leading to FA and triglyceride accumulation. Together with the short-term accumulation of VPA-metabolites 4ene-VPA and 2,4-diene-VPA, the model describes the cell viability impact.

Further, the effect of zonation in liver lobules on a compounds toxicity is investigated using a structured liver module coupled to the PBPK-model (*Figure 4.70*; *Diaz Ochoa et al., 2012; Niklas et al., 2013*). The 1D-liver comprises the transport of the substance both in the sinusoid and into the hepatocytes. Each hepatocyte includes the metabolism of a test compound. This enables the description of a heterogeneous metabolism and phenotype along the sinusoid (zonation). This model was implemented in a KNIME workflow (*Figure 4.73*). The implications of the extension of a PBPK model are reviewed in the literature (*Niklas et al., 2013*).

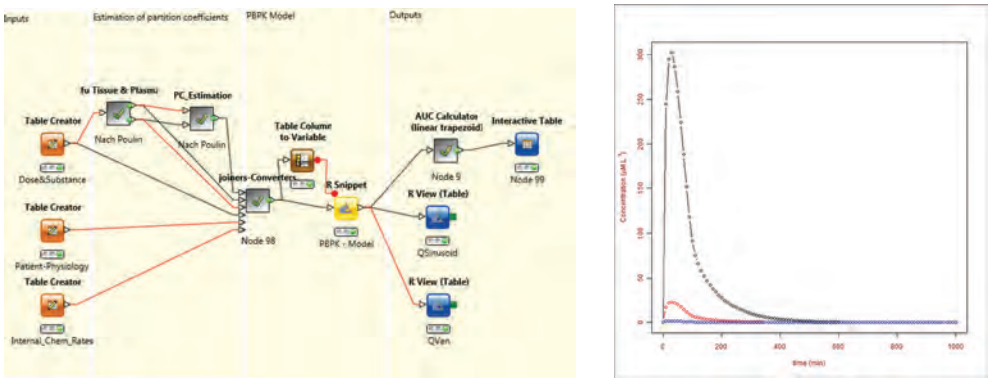

Figure 4.73 *KNIME workflow of the coupled 1D liver. An exemplary mean distribution of acetaminophen concentrations in the liver sinusoid is obtained based on the model of Pery et al. (2013). Black curve - periportal section; red curve - midlobular section; blue curve - pericentral section.*

Conclusions

A model capturing toxic modes of action following the addition of VPA was successfully integrated in a whole-body PBPK model. The cellular model was verified against the conducted VPA multi-scale experiment on HepaRG (see section 4.6.3).

To capture essential liver heterogeneity, a three-compartment liver zonation model with different cellular functionalities in each section was developed, implemented in KNIME and tested for acetaminophen.

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4.9.3.3 PBPK Predicitions of Methotrexate and Valproic Acid Tissue Concentration in Humans

Sophie Teng, Alexandre Péry, Simona Kovarich, Frederic Bois

Introduction

The **SEURAT-1** level 3 *ab initio c*ase study aims to determine the safe dose for a cosmetic ingredient in a consumer use scenario (details about this case study can be found in section 3.5.2). This implies extrapolating the *in vitro* dose-response to *in vivo* effects, on the basis of

target tissue concentrations. Predicting such concentrations is best performed with the help of physiologically based pharmacokinetics (PBPK) models. PBPK models group the organs of a human body into compartments relevant for the kinetics of the compound of interest. These compartments are connected by the blood stream and characterised by physiological parameters such as volume, blood flow, partition coefficient, *etc.* We adapted PBPK models for two **SEURAT-1** standard reference compounds: valproic acid and methotrexate. We show here predictions of tissues concentrations in humans, following clinical dosing.

Methods

The PBPK model includes 4 compartments: adipose tissue, liver, poorly and well perfused organs. This model has been implemented in R using the deSolve package (*Setzer et al., 2010*). The model parameters were set using QSAR predictions (*Rodgers & Rowland, 2006; Peyret et al., 2010; Zientek et al., 2010*) or published *in vitro* metabolism data (*Argikar & Remmel, 2009*).

For methotrexate we did not find QSAR or *in vitro* based estimates of clearance and two scenarios were considered: (i) no clearance; and (ii) blood flow limited hepatic clearance. Confidence intervals were obtained for the predicted concentrations by Monte Carlo simulations. The PBPK predictions for both valproic acid (VPA) and methotrexate (MTX) were compared to published human *in vivo* data (*Nitsche & Mascher, 1982; Perucca et al., 1978; Hroch et al., 2008*).

In order to identify the most sensitive parameters of the models, a sensitivity analysis has been performed on the basis of the Monte Carlo simulations.

Results

The MTX PBPK model fits quite well the *in vivo* data whereas the VPA model under-predicted them after both single and repeated oral dose (*Figure 4.74* and *Figure 4.75*, respectively).

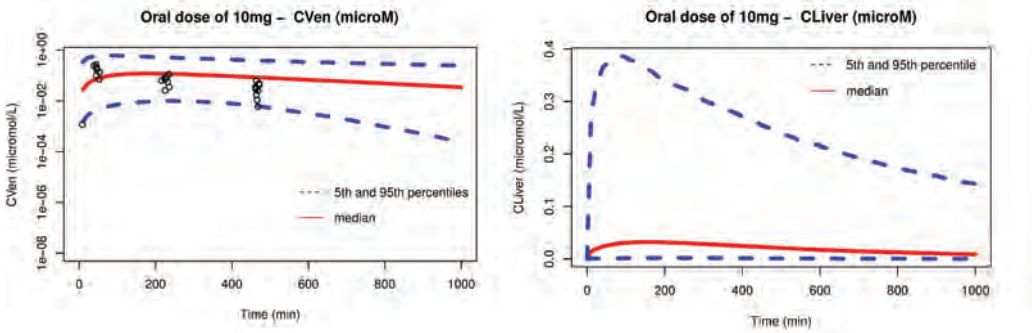

Figure 4.74 *Predictions of methotrexate concentrations in human venous blood (left panel) and in liver (right panel), following a single dose administration of 10 mg.*

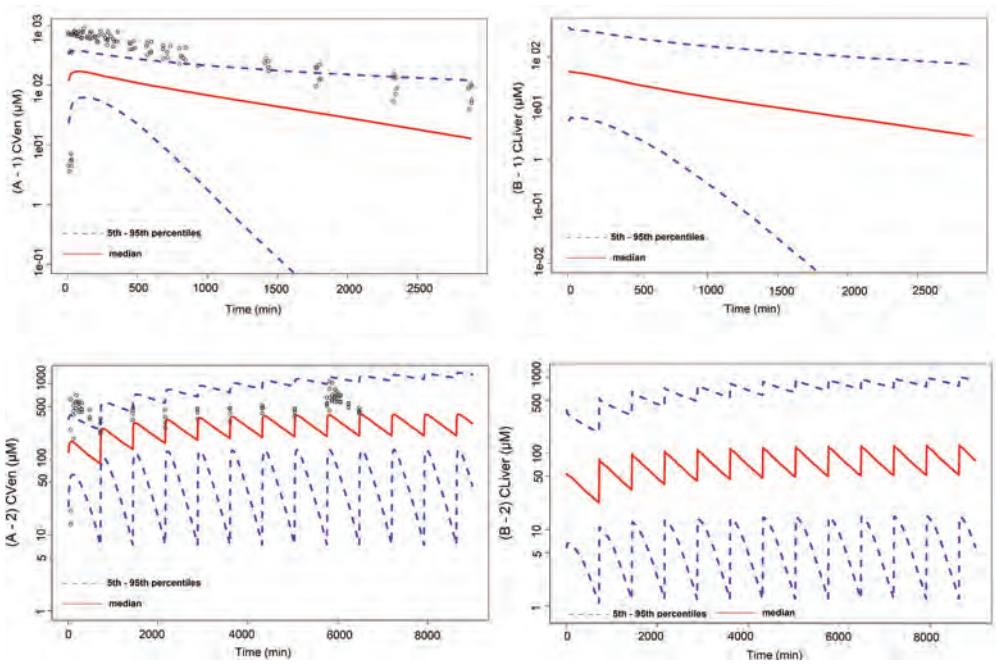

Figure 4.75 *Predictions of valproic acid concentrations in human venous blood (left panels), and liver (right panels), following single oral dose (upper panels A–1 and B-1) and two daily doses (bottom panels A-2 and B-2) of both 1000 mg.*

For MTX, the sensitivity analysis showed in both, venous blood and liver, that the unbound fraction and the bioavailability of MTX were the most sensitive parameters. However in the liver, the liver partition coefficient and hepatic clearance are also sensitive for the hepatic concentration prediction (*Figure 4.76*).

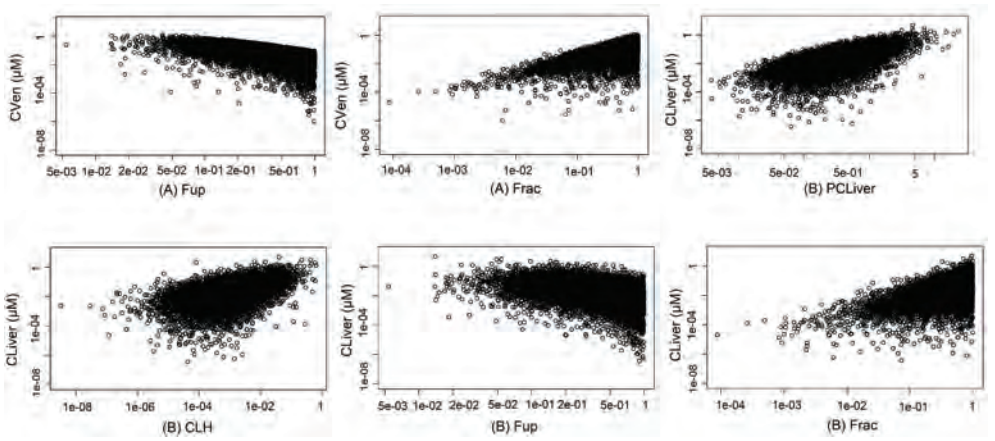

Figure 4.76 *Prediction of (A) venous blood and (B) liver concentrations using 10,000 Monte Carlo sampling of the PBPK model's parameters y (Fup represents the unbound fraction in the plasma; Frac the bioavailability; PCLiver the partition coefficient in the liver and CLH is the hepatic clearance).*

For VPA, only the unbound fraction was the most sensitive parameter in both venous blood and liver. In the liver, the partition coefficient of VPA and hepatic clearance were also sensitive parameters for prediction of the hepatic concentration (*Figure 4.77*).

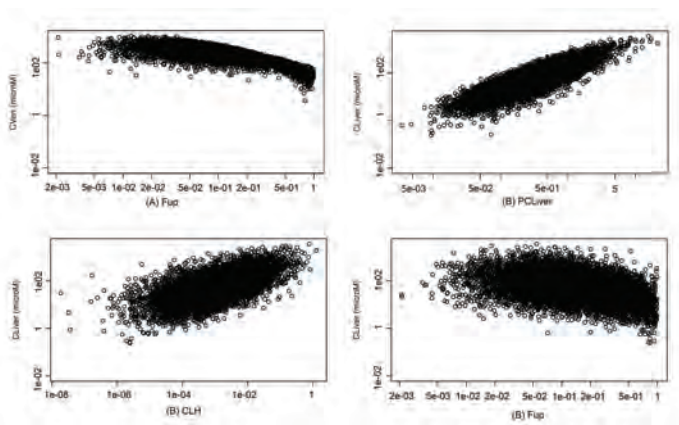

Figure 4.77 *Predictions of venous blood (upper left panel) and liver concentrations (upper right and bottom panels) using 10,000 Monte Carlo sampling of the PBPK model's parameters for the repeated dosing regimen (Fup represents the unbound fraction in the plasma; PCLiver the partition coefficient in the liver and CLH the hepatic clearance).*

Conclusions

In the context of an *ab initio* study, the VPA model under-predicted liver concentrations after single dose oral administration. However, for MTX single dosing and VPA repeated dosing, the *in vivo* data fell within the 90% confidence interval of the model predictions. Additional *in vitro* data on the most sensitive parameters (the unbound fraction, liver to blood partition coefficient, liver clearance and the bioavailability) should improve the model predictions, which is particularly needed for single dose VPA administration.

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4.10 Cross-Cluster Cooperation

4.10.1 The SEURAT-1 Roadmap

Mark T.D. 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; and the demonstration of proofs-of-concept (PoCs), thus providing a blueprint for expanding the applicability of the research strategy. **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 section 4.10.3) in order to support the design of studies in relation to the three PoC levels. The PoCs identified 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 4.78*) 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** (see below). Once completed, this collection will fulfil the second cluster-level objective (i.e., the development of highly innovative tools and methodologies that can ultimately support regulatory safety assessment).

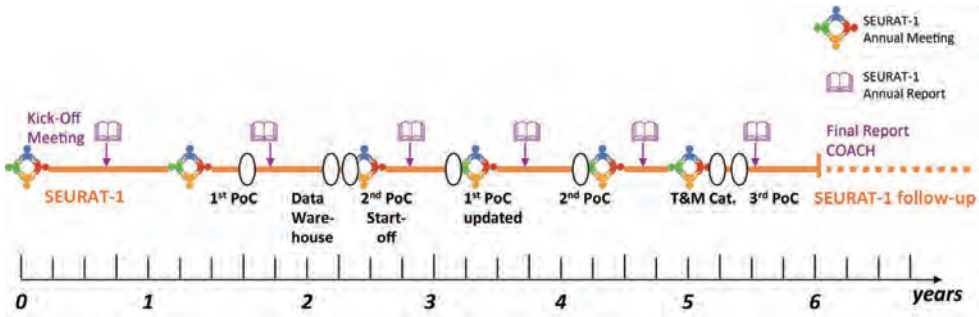

Figure 4.78 *The* ***SEURAT-1*** *timeline.*

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 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 4.79* and demonstrates that the **SEURAT-1** Research Initiative continues to progress along its own roadmap. It is anticipated that reporting of all **SEURAT-1** proof-of-concept case studies will be completed by the end of 2015.

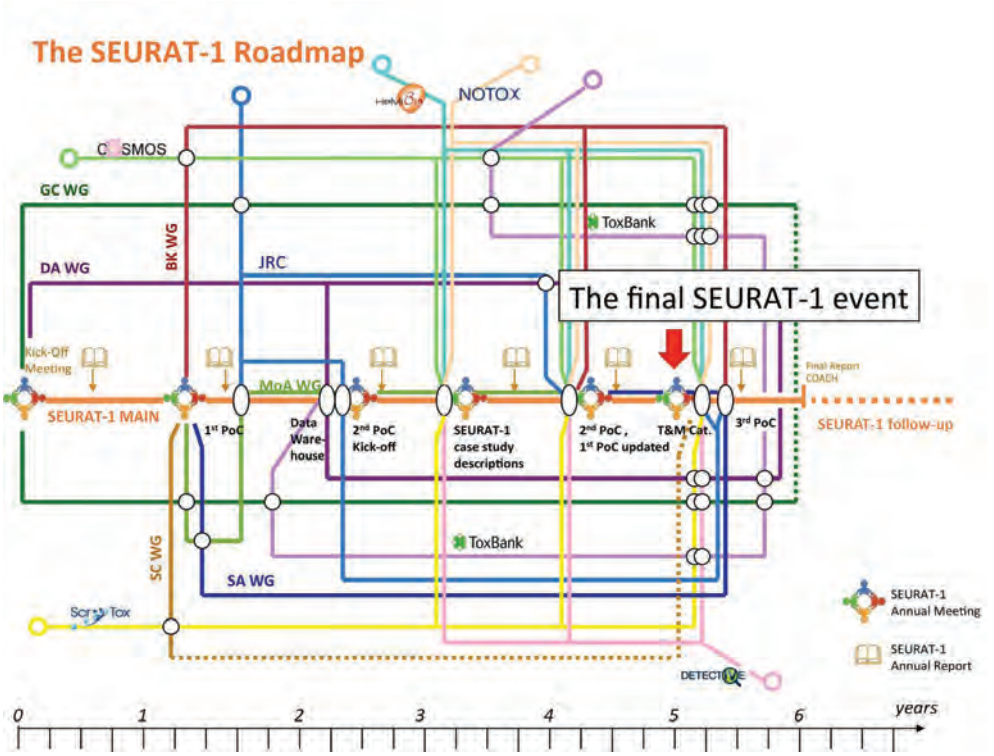

Figure 4.79 *The* ***SEURAT-1*** *roadmap illustrating the contributions from the projects, the Joint Research Centre and the Working Groups to the cluster-level objectives. GC WG = Gold Compound Working Group, DA WG = Data Analysis Working Group, BK WG = Biokinetics Working Group, MoA WG = Mode-of-Action 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.

4.10.2 The SEURAT-1 Tools & Methods Catalogue

Elisabet Berggren

One of the most valuable outputs of **SEURAT-1** will be the large portfolio of cutting-edge computational and *in vitro* tools and methods that will underpin new animal-free approaches to safety assessment. Many of them are already being applied in the **SEURAT-1** case studies to demonstrate their utility in predicting toxicity and supporting decision-making (see chapter 3 of this Annual Report). It is, therefore, important to clearly present the new tools and methods

in their own right, as independent building blocks that can be assembled in a wide variety of ways for many different purposes. To this end, we will develop the ***SEURAT-1*** *Tools & Methods Catalogue*, to be finalised by the end of 2015.

The idea is that the ***SEURAT-1*** *Tools & Methods Catalogue* only includes non-confidential information in a summarised and easy-to-read format – something that anyone can pick up and consult to see what is available. If there is interest in a particular method then more detailed information can be retrieved from the ToxBank Data Warehouse (https://services.toxbank.net/). The catalogue will also serve to showcase the variety, richness and novelty of **SEURAT-1** products and in time will become a recognised stepping stone to modern safety assessment based on alternative methods.

The **SEURAT-1** Scientific Expert Panel agreed in May 2014 that catalogued **SEURAT-1** methods should also be disseminated via the public EURL ECVAM DataBase service on Alternative Methods (DB-ALM; http://ecvam-dbalm.jrc.ec.europa.eu), developed and maintained by the European Commission's Joint Research Centre. The DB-ALM provides standardised curated descriptions of over 300 alternative methods intended for use both in biomedical research and regulatory safety assessment.

Benefits of including **SEURAT-1** methods in DB-ALM include:

- Dissemination to a large, well-established international DB-ALM user community;
- Complementary information in both DB-ALM and ToxBank – higher level method descriptions in DB-ALM will be linked virtually to more technical (protocol) descriptions and related experimental data in ToxBank;
- Publically accessible information in DB-ALM will be separated from any confidential information kept in ToxBank;
- Method descriptions in DB-ALM can be updated at any time, including in the future when **SEURAT-1** is complete;
- Describing a method in DB-ALM can facilitate eventual submission to EURL ECVAM for validation, as a step towards regulatory acceptance;
- Method descriptions in the Catalogue will be derived from those in DB-ALM, so there is no need to provide the same information twice.

The **SEURAT-1** partners are currently providing the DB-ALM with their methods descriptions, and the intention is to distribute the ***SEURAT-1*** *Tools & Methods Catalogue* at the final **SEURAT-1** event in Brussels on 4 December 2015.

4.10.3 The Model of Cross-Cluster Working Groups

The COACH Team

As briefly described in section 4.10.1, 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. *Table 4.5* provides an overview about the **SEURAT-1** Working Groups, including short descriptions (more detailed working group reports are given in the following sections).

Table 4.5 *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 was 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 collaborated on the discussion of compound selection, mechanisms and assays. A criterion for the compound selection was 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 (DAWG) holds ongoing 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 also contributes 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 assisted 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 framework approach was used as a practical tool to organise MoA information and capture interrelations in the cell by means of '-omics' and *in vitro* data, including dose dependencies. A special focus was made trying to link molecular initial events to possible adverse outcomes.
Biokinetics	Frédéric Bois (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 and case studies 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 was to standardise quality control issues of the cells used between different partners and projects. Three cross-consortia cell model subgroups have been identified: pluripotent stem cell lines (DETECTIVE, *SCR&Tox*), embroyd bodies (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 safety assessment decision-making needs. Future safety assessment approaches should be based on comprehensive knowledge of the modes-of-action and pathways leading to adverse effects in humans, rather than on animal testing. This 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.

4.10.4 Gold Compounds Working Group: Mechanism-based Selection of Reference Compounds for the Development of *in vitro* Toxicity Testing Methods

Jeffrey Wiseman, Paul Jennings

Mechanistic understanding of chemical induced perturbation is at the heart of the much heralded paradigm shift in toxicology. Toxicogenomics and complementary '-omic' techniques have accelerated the discovery and delineation of a plethora of pathways which can aid in understanding mode of action of chemical toxins (*Jennings et al., 2013; Wilmes et al., 2013*). To expand this knowledge and to investigate the quality of the *in vitro* systems we are developing in **SEURAT-1** and beyond, we need to test more compounds. The question is which compounds should we test and why? The Gold Compound Working Group, co-chaired by the authors of this section, made a decision early in the project that **SEURAT-1** should focus on compounds with very well defined molecular mechanisms. The rationale is simple: if we know the mechanism of the chemical we are testing, we can assess if the novel *in vitro* systems and surrounding assays are fit-for-purpose. To this end the Gold Compounds Working Group got busy on building chemical lists and dossiers. After much discussions and debates, which are summarised in the previous volumes of this Annual Report, these chemicals were reduced to smaller manageable lists (*Table 4.6*). Information on these compounds is hosted by ToxBank on the compound wiki page (http://www.toxbank.net/compound-wiki).

Table 4.6 *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

In addition, we have supported these actions by conducting an extremely detailed mechanistic review on hepatotoxins and their mechanisms, focusing primarily to these gold compounds (*Jennings et al., 2014*). In the review, we divided compounds into different categories including chemically reactive compounds (alkylating and oxidising agents), compounds with specific cellular targets (mitochondrial toxins and specific enzyme inhibitors), compounds that interfere with lipid metabolism and compounds that disrupt the plasma membrane. We also discuss the molecular and cellular pathways that are perturbed and the cellular responses activated to redress these perturbations. We hope that this information will be valuable for scientists developing *in vitro* models, generating predictive assays and developing adverse outcome pathways.

References

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4.10.5 Data Analysis Working Group: Integrated Data Analysis

Glenn J. Myatt, Nina Jeliazkova, Barry Hardy, Annette Kopp-Schneider

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 discusses best practices, standards and common approaches including topics such as vocabularies, ontologies, statistical analysis, and integrated data analysis. The group also develops ideas and new approaches to data analysis required by emerging research activities carried out

under the programme, such as the choice of biomarkers and approaches to the processing and analysis of associated '-omics' data. In the following, we demonstrate our approach in two scenarios covering the integration of data from external resources to support the analyses of data obtained in **SEURAT-1** projects.

In the last year, there have been on-going efforts to integrate information on external resources with **SEURAT-1** generated information. One of these projects is to integrate the ToxCast (*US EPA, 2015*) and Tox21 (*US Department of Health and Human Services, 2015*) data with the **SEURAT-1** data to support a meta-analysis of the combined information. The first step in this process has been to translate the data files and databases that have been made available by the US EPA into the ISA-TAB format to be uploaded into the ToxBank data warehouse. This includes the generation of information on the overall investigation, the study design as well as each assay that was performed. In addition, the processed endpoints have been standardised based on the harmonisation proposed by the ToxBank consortium. The latest ToxCast/Tox21 release (31 October 2014) contains raw dose response and processed data for 8599 chemical substances and 65 assay endpoints generated by 35 experimental protocols (Tox21 dataset) and raw and processed data for 1877 chemical substances; 821 assay endpoints (outputs), derived from 558 assay components (raw data readouts), generated from 342 experimental protocols (ToxCast dataset). The ToxCast/Tox21 data is organised in 7 levels and it was decided to only include Level5 data at this time (hitc, log AC10, log AC50 gain, log AC50 loss). The ISA-TAB study, assay and data files are generated by SQL queries from the ToxCast MySQL database[1]. In addition to generating the ISA-TAB files from the Tox21/ToxCast data, we are also investigating a setup with ToxCast phase II data hosted in an AMBIT instance (an open-source cheminformatics data management system; *Ideaconsult, 2015*) with the data and structures available via REST API (JSON) to the ToxBank UI to query in the same fashion as it queries the COSMOS database. These approaches are illustrated in *Figure 4.80* showing an example of the ISA-TAB and the access to data from the AMBIT instance.

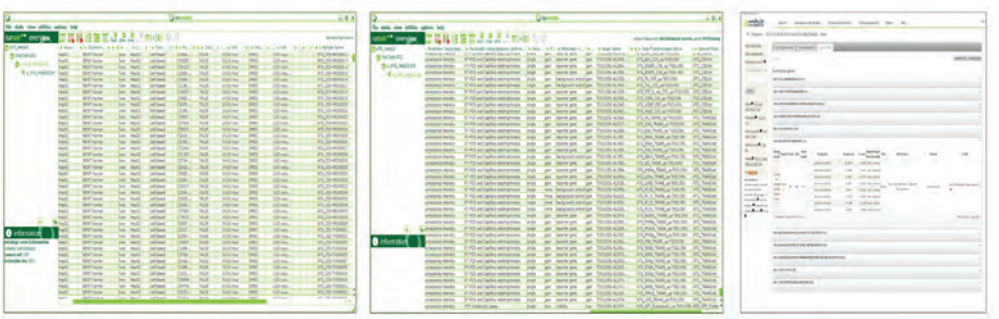

Figure 4.80 *Examples mapping the ToxCast data to ISA-tab and data retrieved from the AMBIT instance.*

1 http://www.epa.gov/ncct/toxcast/files/MySQL%20Database/MySQL_Database_v1.zip

Another project integrating external resources with **SEURAT-1** data is through a collaboration with the DETECTIVE consortium. As part of a publication (*Grinberg et al., 2014*), the TG-GATES transcriptomics data was analysed and the raw and processed data generated was converted to ISA-TAB. In addition, the toxicogenomics directory was made accessible through a wiki page (*ToxBank, 2015*). The ISA-TAB conversion included mapping the processed data onto the standardised format developed by the ToxBank project. To support access to this dataset as well as other '-omics' data in an integrated fashion, a new biomaterials search was developed to support the querying of genes (as well as other biomaterials). This search was developed in consultation with other projects to support identifying specific gene of interest from other experiments. The workflow of this study is shown in *Figure 4.81*.

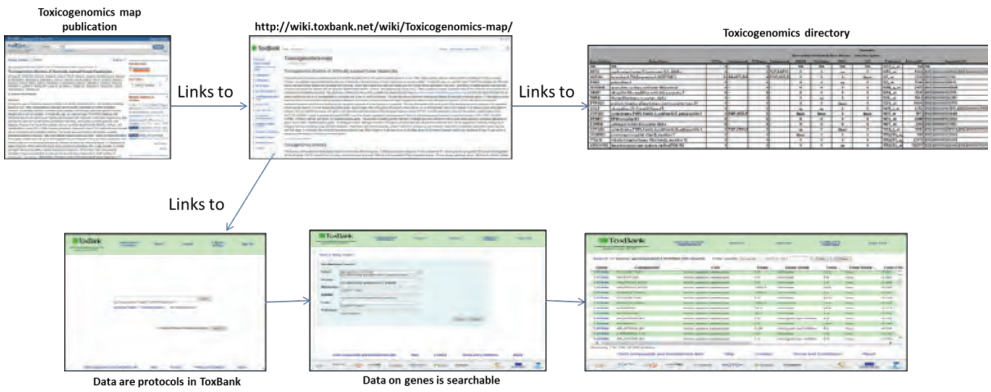

Figure 4.81 *Integration of data from TG-GATES transcriptomics database.*

References

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4.10.6 Mode-of-Action Working Group: Capturing Mode-of-Action Knowledge

Brigitte Landesmann, Mathieu Vinken

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 to 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.

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, ongoing 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 equally strategies to link the MAWG activities to the different **SEURAT-1** projects.

In 2014, focus has been put on the dissemination of activities and the practical elaboration and verification of established AOPs. The latter was mainly done, and is still being done, in the context of the **SEURAT-1** case study programme.

Among the planned activities for this final year of the **SEURAT-1** Research Initiative are:

- Active involvement in the practical performance and evaluation of the proof-of-concept case studies in the different **SEURAT-1** projects.
- Dissemination and communication of MAWG activities on international conferences, including presentation of developed AOPs.
- Generating new relevant AOPs and publication in peer-reviewed journals.
- Further contribution to the development of an AOP knowledge base in collaboration with JRC, OECD and the US-EPA.

- 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.
- Continuing role of 'AOP-ambassadors".

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4.10.7 Biokinetics Working Group: Biokinetic Modelling in Support of *ab initio* Predictions of Safety

Frédéric Y. Bois

Introduction

One of the **SEURAT-1** activities is to explore the potentials and eventual pitfalls of a purely *ab initio* approach to toxicity predictions. Assuming that only the chemical structure of a new molecule is known (along with the relationships between structure and toxicity for all other known molecules, if available), the questions are: can we first predict its absorption, distribution, metabolism and excretion (ADME) by humans? Can we then, on the basis of predicted tissue concentrations relate toxic effects observed *in vitro* (hopefully on human cells) to expected *in vivo* effects?

This summary report addresses the first question, the prediction of ADME and tissue concentrations. The best tools for these aims is a combination of predictive chemistry (e.g., QSAR's) and physiologically based pharmacokinetic (PBPK) modelling (*Adler et al., 2011*), as currently used by the pharmaceutical industry for guessing first dose to human, or for predicting the bioavailability of virtual molecules. However, PBPK modelling must be extended for cosmetic ingredients with dermal exposures and this requires validation datasets for known compounds. For that purpose we used methotrexate (MTX) and valproic acid (VPA), for which both pharmacokinetics and toxicity in humans are known. Arguably, these are not standard cosmetic ingredients, but we played the 'game' of considering them as entirely new molecules, for which we knew nothing, except their molecular structure (and *in vitro* toxicity on hepatocytes, but we focus here on pharmacokinetics). Therefore, in the following, the human data available were only used for checking, or validating, the PBPK model predictions, but not for setting ADME parameter values.

PBPK Models: Parameter Values and Uncertainty Analyses

We used the generic PBPK model developed in the framework of COSMOS. Briefly, the model includes distribution in the following tissues or organs: Venous blood, arterial blood, fat tissues, poorly perfused tissues (muscles), richly perfused tissues (viscera), gut, liver, and skin. Exposure can occur through the dermal route, ingestion or inhalation. The absorbed molecules can be excreted to urine or metabolised in liver. The model is coded as a set of ordinary differential equations, solved by numerical integration with the R 'deSolve' package. The scripts corresponding to the MTX and VPA implementations are available as KNIME workflows on the COSMOS web-portal.

While the structure of the PBPK model used and the list of its parameters are fixed, its parameter

values were tailored to the molecule of interest (MTX or VPA), to the extent permitted by the sole knowledge of chemical structure and eventual *in vitro* tests of ADME properties. We were faced with two cases: For some parameters (*e.g.*, the partition coefficients, the total body clearance) we had *a priori* information from QSAR models (*ACD/LABS, 2015*) or *in vitro* experiments (even if very imprecise), while on others (in particular on the fraction metabolised) we had no information at all. Such uncertainties should be accounted for. That is why we decided to assign a statistical distribution to each model parameter, rather than a single value, and to perform Monte Carlo simulations (*Bois et al., 2010*) to estimate the impact of such parameter uncertainty on model predictions (*e.g.*, on the concentration of MTX or VPA in liver at any given time following exposure). For informed parameters we used distributions with a CV of 300%. For uninformed parameters we used uniform distributions between very wide bounds (*e.g.*, from 0 to 1 for the fraction absorbed by the gut or the fraction unbound in plasma). The correlation between partition coefficient estimates was taken into account. Monte Carlo simulations proceeded by sampling one random value (out of its assigned distribution) for each model parameter. The model was then run and the outputs (predictions) recorded. Those two steps were iterated many times, and the collected output values formed a random sample, for which we computed the mean, the SD, and any percentile of interest. In short, Monte Carlo simulations give predictions' uncertainty provided that they are fed with parameter distributions reflecting uncertainty (or variability).

The following results were obtained that way and present not only the average (mean) predictions but also their 'confidence' intervals (in this case delineated by the 2.5th and 97.5th percentiles).

Results

Methotrexate: In a purely predictive framework we would just obtain predictions such as those given in *Figure 4.82* for liver (right panel) after a 10 mg oral dose. The uncertainty is large, and the upper 95% confidence bound lies one order of magnitude away from the median at the peak level. Simulations of repeated dosing (once a week) indicate no accumulation over time and the patterns shown on *Figure 4.82* repeat themselves every week. In the case of the venous blood (*Figure 4.82*, left panel) we have clinical data (*Stewart et al., 1990; Kozloski et al., 1992; Hroch et al., 2008*), which we can compare to the simulations. Those data were obtained at different doses (7.5, 10, and 15 mg). Since the model assumed linearity, the 7.5 and 15 mg data were simply rescaled by a factor of 0.75 and 0.67, respectively. The data fall within the 95% confidence limits and the decay slope is reasonable, but the peak blood concentration is underestimated by the median predictions. Some Monte Carlo predictions (grey curves) actually match the data quite well, indicating that more precise parameter estimates would probably improve our model predictions.

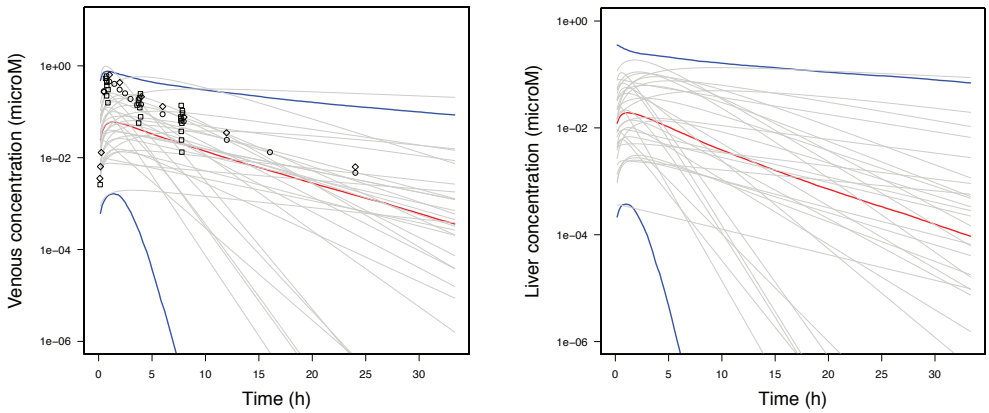

Figure 4.82 *PBPK model predictions of methotrexate venous (left panel) and liver (right panel) concentration in a human adult following a 10 mg ingestion. Red lines: median predictions; blue lines: 2.5 and 97.5 percentiles; grey lines: 20 random simulations. The comparison with measured concentrations is possible for venous blood (squares represent data from Hroch et al. (2008), circles represent data from Kozloski et al. (1992) and diamonds represent data from Stewart et al. (1990).*

If we want to improve the precision and accuracy of our model predictions we need to decide which parameters should be better estimated in priority (obviously we can decide to improve all parameter estimates if we have no time or budget constraints). Monte Carlo simulations can help us here too. Since sampled parameter values were recorded along with the predicted concentrations, it is easy to draw the plots shown on *Figure 4.83*. They show the relationship between the venous concentration of MTX, two hours after dosing (close to the peak time) and the values sampled for three parameters: the fraction absorbed by the gut, the absorption rate and hepatic clearance (actually renal clearance is as important as hepatic clearance). We have seen that the peak concentration is somewhat underestimated (*Figure 4.82*). Narrower ranges for those parameters would also narrow and improve the range of predicted concentrations. Note that the exercise is biased since we have human data which guide the way to improve the model predictions. In a purely predictive framework, we would have to decide which parameters to focus on, just on the basis of *Figure 4.83*. Because we care about the upper bound on peak concentrations we would focus on clearance, on the fraction absorbed and then on the absorption rate. The partition coefficients are less influential (data not shown).

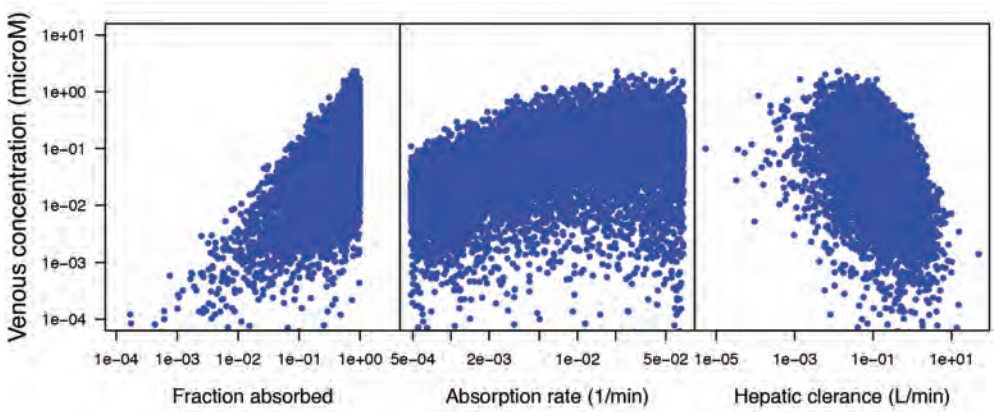

Figure 4.83 *Sensitivity analysis regarding three important parameters affecting the predicted methotrexate venous concentration in a human adult, 2 hours after a 10 mg ingestion. Each dot corresponds to the result of a Monte Carlo simulation (parameters were randomly sampled jointly).*

Valproic Acid: VPA would not accumulate when administered once a week. However, for therapeutic reasons, it is administered once or twice a day and a moderate accumulation is observed. Since we wanted to compare the predicted kinetics with clinical data, we simulated time courses over one week.

Figure 4.84 shows the model predictions for venous blood and liver VPA concentrations during repeated oral dosing (1000 mg every 12 hours). For venous blood, clinical data are available obtained at different doses (800, 900, and 1000 mg; *Perucca et al., 1978; Nitsche & Mascher, 1982*). Since the model is linear, the 800 and 900 mg data were rescaled. The lower bound uncertainty is large, but overall the uncertainty is lower than for MTX. The venous blood data fall within the 95% confidence limits and are reasonably well approximated by the model.

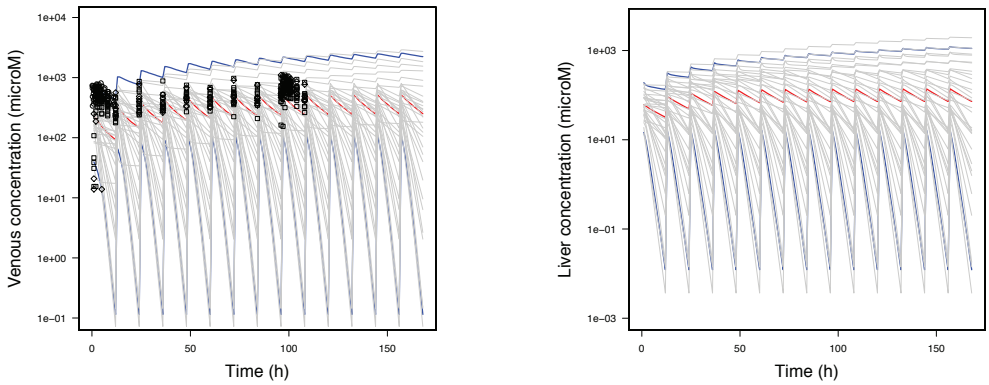

Figure 4.84 *PBPK model predictions of valproic acid venous (left panel) and liver (right panel) concentration in a human adult during repeated oral dosing (1000 mg every 12 hours). Red lines: median predictions; blue lines: 2.5 and 97.5 percentiles; grey lines: 20 random simulations. The comparison with measured concentrations is possible for venous blood (squares and diamonds represent data Nitsche & Mascher (1982) with doses of 900 and 1000 mg, respectively; circles represent data from Perucca et al. (1979)).*

The sensitivity of the predicted VPA venous blood concentration (at 100 hours, that is 2 hours after the 8th 1000 mg dose) is illustrated in *Figure 4.85* for three parameters: the fat - blood partition coefficient, the fraction absorbed by the gut, and the hepatic clearance rate. All partition coefficients are correlated, hence their estimates improve together. Getting a more precise estimate of either hepatic (shown here) or renal clearances would also narrow down our confidence intervals. Here we show the sensitivity to the fraction absorbed. For VPA, that fraction is rather well identified and within its plausible range the predictions are not very sensitive to these values. It would not make much sense to try to improve the estimate by further experiments or calculations.

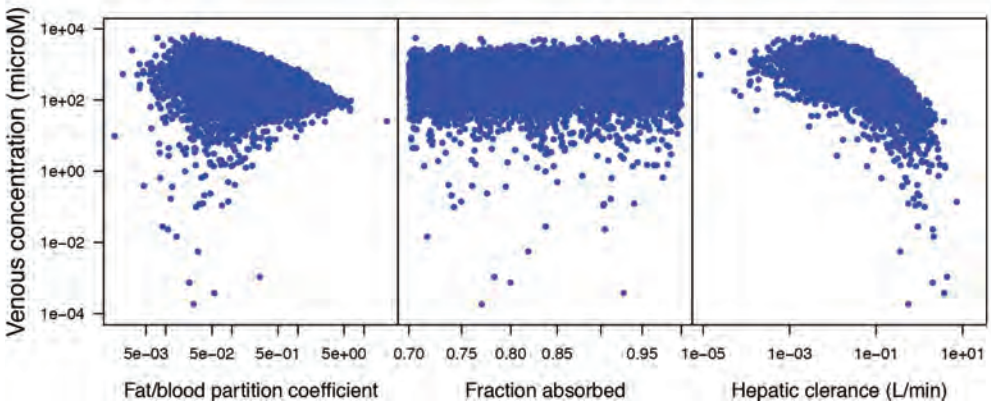

Figure 4.85 *Sensitivity analyses of three parameters relevant for the prediction of valproic acid venous concentration in a human adult, 2 hours after the 8th administration of 1000 mg per os. Each dot corresponds to the result of a Monte Carlo simulation (parameters were randomly sampled jointly).*

Next Steps

A third chemical, piperonyl butoxide, more relevant but less informed than MTX and VPA, is being investigated in the **SEURAT-1** *ab initio* case-study (see section 3.5.2) and the PBPK model will be used to predict the fate of this compound as well. The model can be used in the context of risk assessment: Given experimental *in vitro* dose-response data on liver effects, for example, we can now predict human *in vivo* liver dose for any consumer exposure scenario, and hence *in vivo* effects. We could also refine our parameter estimates prior to deciding a mode of action based on risk predictions, if such predictions are deemed too uncertain. Bayesian decision analysis can help to create such risk management strategies. The results of the Monte Carlo simulations we performed are directly usable in a Bayesian context.

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4.10.8 Stem Cell Working Group

Glyn Stacey, Anna Price

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 stem cell-based assays for predictive toxicology purposes across the **SEURAT-1** projects. It has had particular close interactions with *SCR&Tox* scientists and outputs on good practice have been posted on the ToxBank website.

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 a range of potential 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 developing rapidly but yet to reach regulatory approval in product safety testing. This complex matrix of different cells and methods makes it very difficult to draw comparisons across work in different laboratories and thus standardisation is very challenging.

Overview of Activities

In the last year, the group compiled considerations regarding best practices on core toxicology assay procedures relating to the preparation, storage and use of test and control compounds. The latter activity on toxicology procedures will be presented, along with other educational content on the ToxBank Data Warehouse. Key areas of consideration common to all partners in the **SEURAT-1** Research Initiative when sourcing tissues and cells were addressed and documents describing best practices were published via the ToxBank wiki on Biological Materials (*ToxBank, 2015*). This and other best practice documents on the TixBank website provide templates against which an assessment can be made of the suitability of the biological material for partner projects. These activities were also included in a book chapter on quality control for hPSC lines and the development of hPSC-based assays, that is planned to be released in September 2015 (*Stacey et al., 2015*). Furthermore, the group has revisited the GCCP guidance (*Coecke et al., 2005*) with respect to stem cell culture and assay development standardisation and is coordinating with JRC activities to publish an updated guidance for stem cell lines.

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4.10.9 Safety Assessment Working Group

Derek J. Knight

Introduction

SEURAT-1 delivers computational and experimental tools and related knowledge that are critical components in predictive toxicology approaches. The proof-of-concept exercise to demonstrate the potential of these tools when assembled in an integrated manner is at the theoretical, methodological and application levels by means of case studies. The Safety Assessment Working Group (SAWG) is to bridge the gap between knowledge and tools from **SEURAT-1** and practical safety assessment decision making needs of risk assessors. The SAWG has facilitated the three case studies at the proof-of-concept application level, described in detail elsewhere (see sections 3.5.1 – 3.5.3):

- An *ab initio* risk assessment as a 'stretching target' that will highlight gaps for future research and development and illustrate overall progress made in **SEURAT-1**;
- Using evidence from new-approach methods to improve the robustness of 'read-across' within chemical categories to predict the toxicological properties on 'target' substances from the known toxicology of 'source' substances.
- A safety assessment using the Threshold of Toxicological Concern (TTC) approach.

Conceptual Framework to Combine Evidence

The **SEURAT-1** 'Conceptual Framework' sets out a structure to guide assessors in devising a fit-for-purpose Integrated Assessment and Testing Approach (IATA) that combines information from predictive tools with a stated protection goal. The overall outcome should be robust as it is not based on single pieces of evidence, rather a weight of evidence combined in a biologically-rational manner. The overall assessment results from combing all this information and evidence, including an assessment of the uncertainty associated with the prediction. It may be necessary to improve the assessment if the result is not fit for purpose. The SAWG has been involved in refining the Conceptual Framework (see *Figure 4.86*).

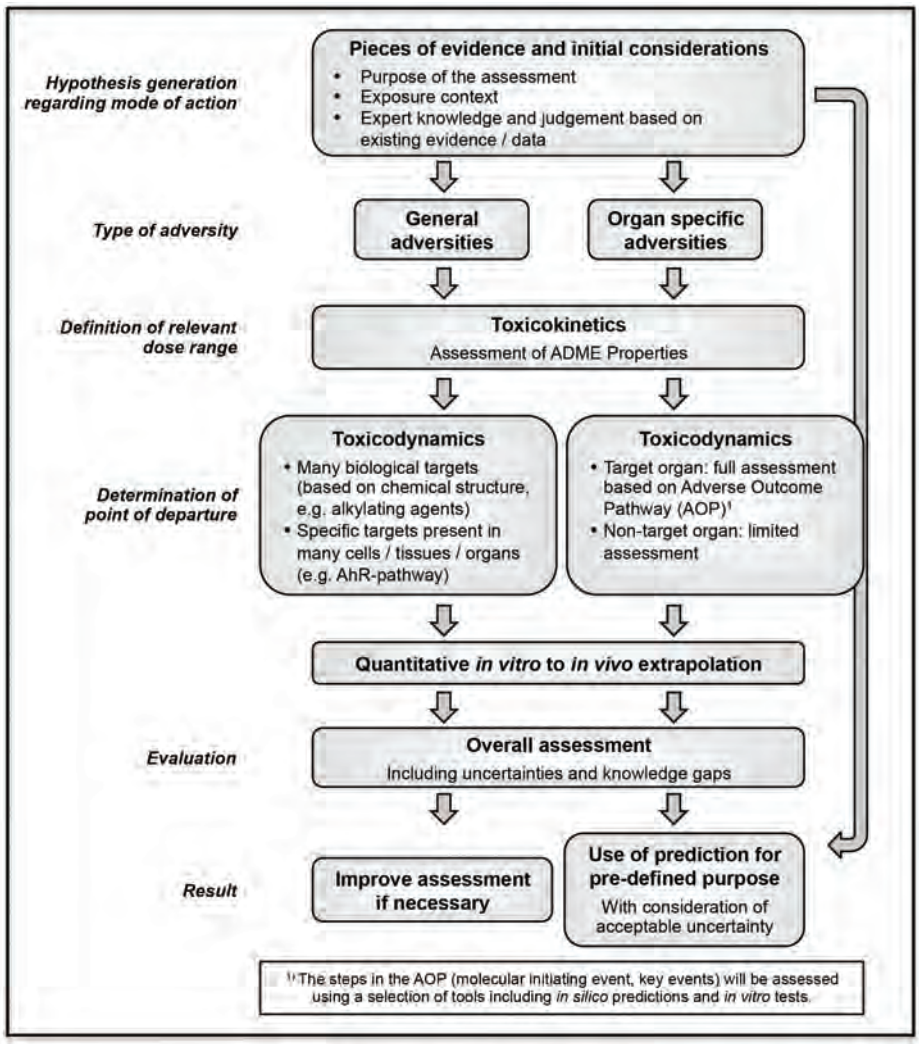

Figure 4.86 *'Conceptual framework' as a structure for assessors in devising a fit-for-purpose 'bespoke' Integrated Assessment Strategy for a particular case (source: Gocht et al., 2015; with modifications).*

Briefly, the Conceptual Framework consists of the following steps:

➠ Decide the degree of confidence needed for prediction (low degree of confidence may be acceptable in case of well-controlled and low human exposure);

- Examine existing knowledge (toxicological studies, 'read-across' from chemical or biological analogues, QSARs and structural alerts, expert judgement);
- Distinguish between 'general chemicals' (expected to be unselective in interacting with biological targets) and a drugs/pesticides (designed to be selectively biologically-active):
- Two parallel lines of consideration: (i) 'general' adverse effects not associated with a particular organ and (ii) organ based adverse effects;
- Consideration of toxicokinetics/toxicodynamics (for both lines of consideration);
- Effects on organs can be assessed by (several) AOPs incorporating existing knowledge and with new data as a combination of *in vitro* assays ('-omics' data etc.) and *in silico* predictions in a battery of tools

ECHA's Topical Scientific Workshop on New Approach Methodologies in Regulatory Science, 19-20 April 2016, Helsinki

ECHA's Topical Scientific Workshops provide a platform for academia and regulators to come together to address important long-term challenges in regulatory science. The idea is to discuss issues framed from the regulatory perspective with scientists who are experts in the field. This bringing together of science and regulatory affairs professionals should help see the topic from different perspectives. The aspiration is that there will arise a better understanding of how to move forward in solving the regulatory challenges. This may be by ideas for better approaches that can be adopted in the short term, when the science is 'ripe'. Equally importantly the discussions will help steer scientific R&D by communicating important regulatory challenges.

The 2016 ECHA Topical Scientific Workshop explores the regulatory application arising from fundamental change in scientific thinking (*ECHA, 2015*). The drivers are a better understanding of the underlying biology behind how chemicals cause adverse effects to human health and new tools and techniques which provide a huge amount of data available from '-omics' and high-throughput screening methods. Complex toxicological apical endpoints cannot be predicted by a single non-standard test; instead it is necessary to combine multiple lines of evidence to predict the hazardous property and tools to facilitate this integration of evidence are integrated assessment and testing approaches with adverse outcome pathways and modes-of-action as the underlying theory. The Workshop draws inspiration from the EU research programme **SEURAT-1** and the US Tox 21 initiative.

The basis of the workshop is to explore using new-approach evidence within the current safety

assessment paradigm of hazard and risk assessment, with a view to possible application in existing regulatory schemes. The central themes reflect the two purposes for assessing properties of chemical substances:

- To assess a specific substance for a defined purpose, e.g. to fill a 'data gap' or to establish safe use from a risk assessment. The central theme is rational combination of data from new-approach methods on an individual substance to make an overall fit-for-purpose prediction for the substance.
- To screen a large set of substances for priority setting to select groups with particular characteristics for further action. The central theme is extracting knowledge from a set of data on a big group of chemicals to make added-value predictions.

Day 1 will focus on all aspects of application new-approach methods and evidence in read-across, including the added value and reduction of uncertainty. Day 2 will cover other means of assessing individual substances and using new-approach methods for screening/priority setting; these discussions will inevitably take place within the context of how to make the most effective use of this new kind of evidence when not restricted to the current regulatory approach (i.e. 'new paradigm' considerations).

Disclaimer

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

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Gocht, T., Berggren, E., Ahr, H.J., Cotgreave, I., Cronin, M.T.D., Daston, G., Hardy, B., Heinzle, E., Hescheler, J., Knight, D.J., Mahony, C., Peschanski, M., Schwarz, M., Thomas, R.S., Verfaillie, C., White, A., Whelan, M. (2015): The SEURAT-1 approach towards animal free human safety assessment. *ALTEX*, 32: 9-24.

4.10.10 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 the first public NOTOX Satellite Meeting finalises this section about cross-cluster collaborations.

1st NOTOX Satellite Meeting – Reaching Out to the Scientific Community

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.

The meeting featured keynote lectures from Dr. Richard Judson, National Center for Computational Toxicology of the U.S. Environmental Protection Agency, and Prof. Mathieu Vinken of Vrije Universiteit Brussel, Belgium, as well as project specific sessions. While Prof. Magnus Ingelman-Sundberg (Karolinska Institutet) presented Novel 3D models for predicting drug toxicity, Dr. Fozia Noor (Saarland University) provided participants with insight into the systems approach in NOTOX case studies. Spatial temporal modelling of *in vitro* and *in vivo* liver tissues was elaborated by Géraldine Cellière (INRIA) and finally, Prof. Elmar Heinzle (Coordinator of the project) presented predictive models for compound testing developed in the frame of the NOTOX project. Moreover, the event featured a NOTOX poster session presenting further relevant aspects of the project. Overall, the event was well appreciated by the participants.

4.11 Training and Outreach

*The **COACH** Team*

4.11.1 Training Activities

Introduction

Training activities have been essential for **SEURAT-1** since the very beginning of the initiative. Although **SEURAT-1** will soon end these activities are getting more attention as a key instrument for transferring newly acquired knowledge to younger scientists at both project- and cluster-level.

At the initiative of COACH a common cluster-level training strategy was developed at the beginning of **SEURAT-1**, going beyond the project-level training activities planned as part of the individual project work-plans. COACH assessed the training activities of the individual projects, developed a proposal for a cluster training concept and initiated the **SEURAT-1** Training Task Force (STTF). The STTF became an important task force of the cluster, meeting annually to discuss implementation of the training strategy in the most efficient way.

The **SEURAT-1** Summer School, held in 2012 in Portugal, was the first accomplishment of the STTF. As planned in the training strategy, 2013 was dedicated to individual project training programmes, focusing on project-level training needs. During 2013, COACH began organising the next cluster-level training activities for 2014 by carefully analysing the feedback from the first summer school. Based on this input, the STTF agreed to take the opportunity to organise a joint summer school in June 2014, as suggested by the European Society of Toxicology *In Vitro* (ESTIV).

SEURAT-1 Summer School 2014

The SEURAT-1 & ESTIV Joint Summer School took place from 8-10 June, 2014, and was linked to the ESTIV2014 conference, which gathered *in vitro* and *in silico* toxicologists from many different countries, representing academia, industry and regulatory bodies. Given that *in vitro* and *in silico* toxicology are cornerstones of the **SEURAT-1** Research Initiative, a considerable overlap in the **SEURAT-1** and ESTIV2014 target audiences was expected. The two events were also intentionally chosen to be held in the same venue: the Zuiderduin hotel, Egmond aan Zee, the Netherlands. This gave summer school participants the unique opportunity to meet toxicology experts attending the ESTIV2014 conference as well as experts from other domains participating in the following two satellite workshops organised in the same hotel: the

NOTOX Satellite Meeting, with focus on a systems biology approach 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.

Close to fifty young researchers, experienced principal investigators and renowned experts from within and outside of **SEURAT-1** attended the summer school (*Figure 4.87*). The objectives of the summer school were to:

- Spread knowledge from the **SEURAT-1** related research domains within and beyond the cluster;
- Provide the opportunity for young scientists to attend practical courses given by eminent experts and to meet colleagues from other research groups to present and discuss their work;
- Gain high visibility of the summer school via the ESTIV2014 conference and allow the participants to network with the ESTIV guests.

Figure 4.87 *Poster session and a lecture during the* ***SEURAT-1*** *& ESTIV Joint Summer School.*

The two day summer school programme was composed of 16 sessions addressing topics from the **SEURAT-1** research areas. To create this programme, all cluster projects first provided a list of sessions, which were then included in a simple questionnaire circulated to the **SEURAT-1** young scientists potentially interested in the event. Based on the questionnaire results, the sessions that received the most votes were included in the final programme. Further to the feedback from the first summer school, the programme was mostly composed of practical courses rather than key-note lectures. These sessions included workshops and discussions, hands-on computer sessions and soft skills courses. A poster session and one social event was also included in the programme to support networking (see *Table 4.7*).

Table 4.7 *Programme of the* ***SEURAT-1*** *& ESTIV Joint Summer School.*

DAY 1: 8 JUNE 2014	
Introduction to Adverse Outcome Pathways (AOPs) for Toxicity: Description, Practical Application and Use	COSMOS
Development of a Liver Bioreactor	*HeMiBio*
In vitro Systems for Metabolism Studies: from S9 to 3D Culture Systems	NOTOX
Epigenetics in *in vitro* Toxicology: Principles, Technology, Data Analyses, and Challenges.	DETECTIVE
Experimental Design of *in vitro* Assays	TOXBANK
Social Event - Bowling	
DAY 2: 9 JUNE 2014	
In vitro to *in vivo* Extrapolation Strategies	Bas Blaauboer (ESTIV)
Toxicological Data Resources: The Basis of Computational Modelling and Safety Assessment	COSMOS
Computational Approaches to Toxicity Prediction: Introduction to Grouping, Category Formation and Read-Across	COSMOS
TTC – for Cosmetics and Beyond	COSMOS
Toxicity Prediction Based on MoA Knowledge	COACH
Pathway Analysis and Visualisation	TOXBANK
Hands-on Training on ToxWiz Software for the Analysis of High-Throughput '-omics' Data	NOTOX
Scientific Methods	SCR&Tox
How to Write a Scientific Paper & Presentation Techniques	COACH
Modelling Toxicokinetics & Toxicodynamics	COSMOS
Poster Session	
DAY 4: 11 JUNE 2014 – EMBEDDED IN THE PROGRAMME OF THE ESTIV CONFERENCE	
Job Opportunities and Career Exploration	ESTIV

The collaboration between **SEURAT-1** and ESTIV provided many benefits for the participants, including:

➠ Free accommodation during the summer school for **SEURAT-1** members that attended both summer school and ESTIV2014;

➠ Free access to the career session organised during the ESTIV2014 conference for **SEURAT-1** young researchers;

➠ The opportunity for intensive exchange between ESTIV2014 participants who arrived earlier to the conferences and attended the summer school poster session. Some of the ESTIV2014 organisers also gave speeches at the summer school.

In addition to the above, COACH launched a number of dissemination activities during this training event aiming to reinforce the **SEURAT-1** visibility:

➠ Dr Emma Davies, a science reporter, was invited to attend the event to get a closer look on the research activities. The knowledge Dr Davies gained during the event was, in her words, extremely valuable, and useful for producing her articles. COACH then decided to pursue this successful collaboration and is currently discussing Dr Davies' further involvement in **SEURAT-1** outreach activities.

➠ A team of students from the Media Competence Center of the University of Tuebingen were invited to the summer school to make a short film about **SEURAT-1**. The event gave them a unique opportunity to interview a number of **SEURAT-1** experts, including Prof. Mathieu Vinken, Dr Alicia Paini and Dr Yaakov Nahmias. The film is now used as a communication and dissemination tool. It was presented at the **SEURAT-1** display (hosted on the JRC stand) at the World Congress in Prague in August 2014 and is available on the **SEURAT-1** website.

Figure 4.88 *Screenshot from the SEURAT-1 video. The video was recorded during the Summer School 2014 and can be watched on Youtube. (https://www.youtube.com/watch?v=FeToRRxMo1A)*

The second **SEURAT-1** Summer School organised in conjunction with ESTIV was a very fruitful collaboration, bringing a new dimension to the cluster training activities. In addition to the scientific discussions and networking between the participants on their ongoing research and development work, the event brought visibility to **SEURAT-1** and allowed for advanced dissemination activities. The feedback received from the participants on site was extremely positive, particularly as they could learn more about the different research domains within **SEURAT-1**.

Other Project Activities

The individual project-level training events continued according to internal needs in 2014 and at the beginning of 2015:

HeMiBio Winter School: The fourth *HeMiBio* winter school '*In vitro* biokinetics' was organised by Catherine Verfaillie, Mathieu Vinken (VUB), Leo van Grunsven (VUB) Aernout Luttun (KU Leuven) and Pau Sancho-Bru (IDIBAPS). It comprised a half-day workshop in Chur, Switzerland on 13 January, just before the *HeMiBio* Annual Meeting. Because of confidentiality issues the workshop was only open to **SEURAT-1** members.

Within *HeMiBio*, different tissue culture set-ups are used that will ultimately be incorporated into 2D and/or 3D microfluidic bioreactors. Currently, different cell types, as well as co-cultures of hepatic cells, are developed as screening tools for the detection of apical endpoint pertinent to repeated dose toxicity, including liver steatosis, fibrosis and cholestasis. Most experiments are still carried out in regular 24/96 well plates, but a 2D bioreactor set-up is used as well. Among the parameters to be addressed during the evaluation of these cell systems and experimental set-ups is the effective concentration at which a certain adverse outcome can be observed. Indeed, final compound concentrations that accumulate inside a cell need to be determined and compared with *in vivo* toxic doses. Several detectors are being developed to monitor a number of read-outs, such as pH, O_2, glucose, lactate, ALT (glutamate), NH_4^+/urea.

The 2015 Winter School highlighted the importance of biokinetics in *in vitro* testing in order to obtain reproducible results in different systems and potentially a correct extrapolation to an *in vivo* dose-response relationship. During the Winter School we learned that processes such as evaporation, protein binding, binding to the plastic well plate, and metabolism can substantially decrease the concentration that was added, resulting in a lower concentration at the precise target in the cells. However, only very few *in vitro* studies address this issue, and the nominal concentrations rather than the actual level of cell exposure is usually taken into account. Calculation of free concentration in the medium or in the cell is not required for all the compounds, thus, decision trees on when it is necessary to calculate free concentration are being developed. Finally, the importance of biokinetics in the context of the *in vitro* systems that are being used within *HeMiBio* and the main limitations of measuring free concentration in a microfluidic setting were discussed.

COSMOS Webinars: A series of COSMOS Webinars were held, hosted by the American Society for Cellular and Computational Toxicology (ASCCT). The first webinar 'COSMOS DB: A New Database of Toxicological Information to Support Knowledge Discovery' was held on 26 February 2014, with the available 125 places fully booked. The 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). The second COSMOS webinar 'Threshold of Toxicological Concern – an approach for safety assessment and its applicability to cosmetics-related chemicals' was presented on 24 July 2014; the slides and a recording are available from http://www.cosmostox.eu/what/ttc. The third webinar 'Automated *in silico* tools for *in vitro* to *in vivo* extrapolation' was held on 13 April 2015; recording and slides will be available at http://www.cosmostox.eu/what/ivive.

ToxBank Training Activities: ToxBank has provided a series of training sessions, both face-to-face through different consortium activities, and as part of the annual meetings and summer schools. In addition, the ToxBank consortium has regular online seminars and meetings to support the use of the ToxBank data warehouse.

4.11.2 Workshops

Within **SEURAT-1** a number of workshops were held and are currently being organised by the different Working Groups (see sections 4.10.4–4.10.9). Particular attention was given to the case studies and COACH organised two workshops addressing the application of the methods developed within **SEURAT-1** in the regulatory context: the read-across case study reported in section 3.5.1 and the *ab initio* case study reported in section 3.5.2.

In addition to these, several workshops took place outside the Working Group activities to address specific aspects of repeated dose systemic toxicity. Respective reports are given in section 4.10.10.

4.11.3 Conferences

9th World Congress on Alternatives and Animal Use in the Life Sciences

The fourth volume of the **SEURAT-1** Annual Report was launched at the 9th World Congress on Alternatives and Animal Use in the Life Sciences (WC9) in Prague, Czech Republic, in August 2014 (*Figure 4.89*). A number of promotional and dissemination activities accompanied the book launch, ensuring high visibility of **SEURAT-1** at this major event in the field of alternative methods.

Figure 4.89 *Launch of the 4th* ***SEURAT-1*** *Annual Report at the 9th World Congress on Alternatives and Animal Use in the Life Sciences in August 2014 in Prague, Czech Republic.*

COACH, together with the SEP, elaborated a detailed plan of all activities at the WC9 that was distributed to all **SEURAT-1** members in advance. It included the following:

- A **SEURAT-1** display within the JRC ECVAM booth available to all WC9 visitors during the entire congress (*Figure 4.90*). Its main objective was to attract stakeholders and other participants interested in the research initiative, but also those ignoring its existence so far, and inform them about the **SEURAT-1** activities via direct discussions or promotional material to take away, including leaflets, USB keys, Annual Reports, roll-up banners, and the COACH film. Discussions and bilateral interviews between visitors and key **SEURAT-1** members attending the WC9 were encouraged to enhance interactions and networking. The stand was also a strategic meeting point for all members of **SEURAT-1** who attended the event.

- A number of scientific sessions as part of the official WC9 programme were related to **SEURAT-1**. Catherine Mahony (Procter & Gamble) gave an overview presentation about the **SEURAT-1** Research Initiative, giving the participants a comprehensive understanding of the goals and first achievements of the initiative. Michael Schwarz (University of Tuebingen) chaired a session on repeated dose systemic testing with speakers from **SEURAT-1** and other initiatives. In addition, all speakers presenting research activities related to **SEURAT-1** advertised the **SEURAT-1** display and the Annual Report launch by

including a common slide in their presentations.

➠ The official **SEURAT-1** Annual Report launch took place at the JRC booth on 26 August 2014. It appeared to be a great success with the authors' speech attracting a high number of participants interested in **SEURAT-1**.

➠ **COACH** also took the opportunity to welcome some journalists at the event, benefiting from the presence of many important **SEURAT-1** leaders and other stakeholders active in the field of alternative testing strategies. They were able to arrange interviews with **SEURAT-1** members to get a better understanding about the objectives and scientific advancements of the initiative.

Figure 4.90 *The* ***SEURAT-1*** *corner hosted by the JRC stand at the 9th World Congress on Alternatives and Animal Use in the Life Sciences in August 2014 in Prague, Czech Republic.*

The WC9 was considered a crucial event during the fourth year of **SEURAT-1**, as it allowed for broad dissemination of the cluster progress and its achievements so far. Thanks to the well-elaborated and coordinated approach, **SEURAT-1** gained great visibility within the international scientific community.

Other Conferences

Within the period covered by this annual report the **SEURAT-1** Research Initiative was further represented via its projects at a number of international conferences. A highlight was the session at the Society of Toxicology's (SOT) 53rd Annual Meeting, entitled 'Computational Approaches to Predict Repeat-Dose Toxicity: Lessons Learned from Cosmetic Ingredients'. This session was organised by the COSMOS project and took place in a full meeting room on 23 March 2014 in Phoenix, Arizona. The session introduced COSMOS and **SEURAT-1** and included five presentations from COSMOS and SAB members as well as a discussion with the stakeholders present in the audience. The session was summarised on SOT's ToXchange

blog website (http://toxchange.toxicology.org/p/bl/et/blogaid=999&source=1). In addition, seven COSMOS-related posters were presented at the SOT conference.

Further activities on conferences are summarised in *Table 4.8.*

Table 4.8 SEURAT-1 *Research Initiative participation in international conferences and workshops.*

Conference	Date	Place	Contribution	Project
49th Annual Meeting of the European Association for the Study of the Liver	9-13 April 2014	London. UK	Poster	*HeMiBio*
UK-QSAR and ChemoInformatics Group Spring Meeting	29 April 2014	Windlesham, UK	Poster and oral presentations	COSMOS
5th Symposium of the Latin American Society of Toxicologic Pathology	11-14 May 2014	Sao Paolo, Brazil	Oral presentations	*HeMiBio,* DETECTIVE
National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) Workshop	8-9 May 2014	London, UK	Poster	*HeMiBio,* DETECTIVE, COSMOS
4th International Conference on Alternatives for DNT Testing	12-14 May 2014	Philadelphia, USA	Oral presentation	*SCR&Tox*
SELECTBIO's conference ‚Advances in Cellular Assays & Cell Culture'	14-15 May 2014	Barcelona, Spain	Oral presentation	NOTOX
CAAT Workshop	17 May 2014	Baltimore, USA	Oral presentations	*SCR&Tox*
NOTOX Satellite meeting at ESTIV 2014	10 June 2014	Egmond aan Zee, The Netherlands	Poster and oral presentations	*HeMiBio*, DETECTIVE NOTOX
OECD Meeting of the extended advisory group on molecular screening and toxicogenomics	12-13 June 2014	Paris, France	Oral presentation	DETECTIVE
18th International Congress on *In Vitro* Toxicology (ESTIV 2014)	10 - 13 June 2014	Egmond aan Zee, The Netherlands	Poster and oral presentations	*HeMiBio*, DETECTIVE, COACH, NOTOX
16th International Workshop on Quantitative Structure-Activity Relationships in Environmental and Health Sciences (QSAR 2014)	16-20 June 2014	Milan, Italy	Poster and oral presentations	COSMOS
Dechema 3D cell culture meeting	24-27 June 2014	Freiburg, Germany	Oral presentation	NOTOX
Annual Meeting of the European Environmental Mutagenesis and Genomics Society (EEMS)	11 July	Lancaster, UK	Poster and oral presentations	COSMOS
9th World Congress on Alternatives and Animal Use in Life Science	24-28 August 2014	Prague, Czech Republic	Launch and distribution of the 4th SEURAT-1 Annual Report, oral presentations and posters from all projects	All

42nd Annual Meeting of the European Teratology Society	2 September 2014	Hamburg, Germany	Poster and oral presentations	COSMOS
NIH workshop on Adverse Outcome Pathways	3-5 September 2014	Bethesda, USA	Oral presentation	DETECTIVE
49th Congress of the European Societies of Toxicology (EUROTOX)	7–10 September 2014	Edinburgh, Scotland	Poster and oral presentations	*HeMiBio*, DETECTIVE, NOTOX, COSMOS
16th Congress of the European Neuroendocrine Association	10-13 September 2014	Sofia, Bulgaria	Poster	COSMOS
IMEC Academy, School for Biomedical Systems, 'Nanotechnology for Health'	22-25 September 2014	Leuven, Belgium	Oral presentation	*HeMiBio*
Schrödinger 14th Annual European User Meeting	29 September – 1 October 2014	Frankfurt, Germany	Poster and oral presentations	*HeMiBio*, DETECTIVE
7th Annual Scientific Meeting of the Australasian College of Toxicology and Risk Assessment (ACTRA)	8 October 2014	Sydney, Australia	Poster and oral presentations	COSMOS
Workshop on 'Application of –omics data in regulatory toxicology'	9-10 October 2014	Berlin, Germany	Workshop organisation and moderation, presentation	NOTOX
IFSCC 28th International Federation of Societies of Cosmetic Chemists Congress	27–30 October 2014	Paris, France	Poster and oral presentations	COSMOS
3rd Annual ASCCT Meeting	12 November 2014	Bethesda, USA	Oral presentation	COSMOS
IFRA Biannual Meeting	18-19 November 2014	Berlin, Germany	Oral presentation	COSMOS
ESTIV Applied *in vitro* Toxicology Course	25-29 January 2015	Lisbon, Portugal	Oral presentation	*HeMiBio*, DETECTIVE
Regulatory Science Associates (RSA) Conference	28 January 2015	Cheshire, UK	Poster and oral presentations	COSMOS
OpenTox	10-12 February 2015	Baltimore, USA	Organised by Barry Hardy	ToxBank
EFSA WG Epidemiology Meeting	18-19 February 2015	Paris, France	Oral presentation	*HeMiBio*, DETECTIVE
SOT 54th Annual Meeting	22-26 March 2015	San Diego, CA, USA	Workshop Session & ToxBank booth	All
British Toxicology Society 2015 Annual Congress	19-22 April 2015	Solihull, UK	Oral presentation	COSMOS
25th SETAC Europe Annual Meeting	3-7 May 2015	Barcelona, Spain	Poster and oral presentations	COACH
2nd International Conference Of Alternatives to Animal Experimentation	8-9 May 2015	Lisbon, Portugal	Poster and oral presentations	COSMOS
UK-QSAR and ChemoInformatics Group Spring Meeting	21 May 2015	Leeds, UK	Poster and oral presentations	COSMOS

Forthcoming conferences

Many dissemination activities are foreseen for 2015, two of which should be highlighted in particular:

- The Society of Toxicology's (SOT) 54th Annual Meeting: Workshop Session 'Painting the Future of Repeat-Dose Systemic Toxicity Testing: Progress from the European **SEURAT-1** Project'.
- The EUROTOX 2015 Congress: Session 'New approaches to repeated dose toxicity assessment – are we ready to replace animal testing?' Moreover, as at the WC9, a dedicated **SEURAT-1** display will be hosted at the JRC booth.

4.11.4 SEURAT-1 Public Website

The **SEURAT-1** public website (www.seurat-1.eu), one of the first key channels of **SEURAT-1** outreach activities, was launched in 2011. The website disseminates information from the cluster to a large audience of stakeholders, scientists and the general public. It also provides statistics regarding the impact of the dissemination activity.

The content of the website is regularly updated and includes: (i) a general overview of the **SEURAT-1** Research Initiative; (ii) detailed information about the objectives and results; (iii) the initiatives' future vision and strategy; (iv) the work structure and (v) overviews of the seven cluster projects involved and their contributions. New cluster-level and individual projects' dissemination material, such as the COACH film, is published immediately on the 'Publications' webpage; the 'Who-is-Who' webpage is now a well-known depository of important information about the experts involved in **SEURAT-1**; the 'Bibliography' webpage contains articles about **SEURAT-1** and the domain of alternative testing in general; and the 'Online library' webpage, launched in early 2015, lists the scientific publications resulting from **SEURAT-1** research work. The website further informs about forthcoming events, training activities and other important news within the cluster.

The following figures (*Figure 4.91* and *Figure 4.92*) give an overview of the visits on the public website for the period from April 2014 until March 2015. The two busiest periods in terms of site visitors were during the WC9 and the Annual Meeting events.

	Country	Sessions	% Sessions
1.	France	3,745	14.16%
2.	Germany	3,599	13.61%
3.	United Kingdom	3,074	11.62%
4.	United States	2,638	9.97%
5.	Italy	2,229	8.43%
6.	Belgium	1,961	7.41%
7.	Brazil	1,001	3.78%
8.	Japan	772	2.92%
9.	Spain	652	2.47%
10.	Sweden	584	2.21%

Figure 4.91 *The* ***SEURAT-1*** *public website statistics for the last year: number of site visitors and visitor countries (source: Google Analytics).*

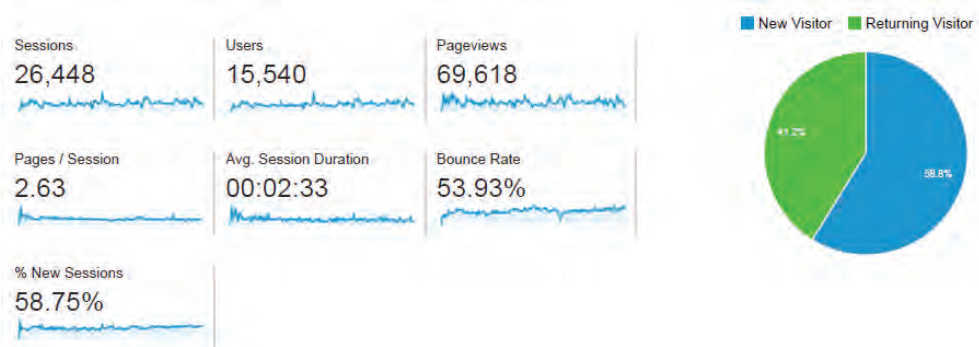

Figure 4.92 *The* ***SEURAT-1*** *public website statistics: total number of visits (source: Google Analytics).*

4.11.5 Other Dissemination Activities

All **SEURAT-1** dissemination material is updated on a regular basis to reflect the progress made. The **SEURAT-1** leaflet with embedded USB drive, **SEURAT-1** and **COACH** posters and the PowerPoint presentations are now a standard part of each conference dissemination package. The USB drive was especially well-received; it is in the form of a credit card and contains all Annual Reports in electronic form. The USB drive is highly appreciated because of its handy format and hundreds of units have been disseminated at international conferences.

An essential dissemination activity was further developed during year 2014 - collaboration with journalists and mass media in general. Indeed, as **SEURAT-1** progresses and more results become available the importance of dissemination activities increases and good media coverage becomes an important objective. **COACH** has therefore initiated a close collaboration with a freelance scientific journalist and a specialised media consultant with the aim to make **SEURAT-1** visible to a wider range of scientific and general mass media (online journals, radio, television, etc.). A special body, Editorial Review Board, was established within the SEP. The board is in charge of carefully assessing the existing dissemination plan and of reviewing the material to be published. The objectives are to:

- Publicise the **SEURAT-1** research initiative, its global objectives and its intermediate and final achievements, adapting the scientific content for the general public;
- Provide visibility and awareness of EU efforts invested in health-related research, particularly in the domain of alternative human safety testing;
- Achieve positive understanding of the aims of **SEURAT-1** and more general research efforts in the domain of alternative human safety testing.

5 PREPARING FOR THE FUTURE

"Nothing is less predictable than the development of an active scientific field."

Charles Francis Richter

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 to fully replace of animal testing in the area of repeated dose systemic toxicity within the lifetime of the research programme. 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 also currently active. This chapter will provide an overview of 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. In the previous Annual Report we highlighted the collaboration with the related initiative in the USA, Tox21, which was set up in 2013 and culminates now in the involvement of Tox21 researchers in the **SEURAT-1** read-across case study (see section 3.5.1). In the last year the focus was to explore options for collaborations with the IMI MIP-DILI project (see report in section 5.3) and, as the **SEURAT-1** Research Initiative moves towards its end, the idea behind such collaborations is now to make other, related initiatives aware of **SEURAT-1** resources that they can use in their projects. This is particularly true for a programme that will be funded by the European Commission's funding scheme, Horizon 2020, which is expected to follow-up the efforts of the **SEURAT-1** Research Initiative and is briefly described in the following section. At the time of writing this report the selection of the successful proposal is on-going and one of the major tasks of COACH, which will run until the end of 2016, will be to support the new consortium during the initial phase and hand-over. The **SEURAT-1** Research Initiative will leave a solid foundation, on which future initiatives can build further activities. Some considerations about how to proceed based on the achievements of **SEURAT-1** were published at the beginning of 2016 and are summarised in the final section of this fifth **SEURAT-1** Annual Report (see section 5.4).

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 2014 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 current 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 are addressed, and the area of 'Health, Demographic Changes and Wellbeing' was identified as one of seven 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 is key to the success of applications for funding under Horizon 2020, as this will establish a new focus on innovation-related activities that bridge 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 seven societal challenges. The call for proposals that was most relevant to the **SEURAT-1** Research Initiative was published within the work programme of the societal challenge 'Health, Demographic Change and Wellbeing':

PHC 33 – 2015: New approaches to improve predictive human safety testing (within the area 'Improving health information, data exploitation and providing an evidence base for health policies and regulation): Proposals should focus on approaches that improve the efficiency of predictive toxicological testing to address key areas of concern for human health and to meet regulatory requirements. Proposals 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. The objectives are:

- To develop and validate routine, non-animal approaches for toxicity testing of chemical substances (excluding radio-chemicals);
- To develop methodologies for confirmatory testing of mechanistic hypotheses to improve understanding of toxicity mechanisms.

International cooperation has been identified as key to success in research and innovation and, consequently, cooperation was encouraged with similar initiatives in the USA and elsewhere. The expected impact comprises the following issues:

- More effective, faster and cheaper toxicological testing to better predict human risk and meet regulatory needs;
- Improved toxicological knowledge to encourage 'read-across' between chemical substances for use in different research and regulatory domains;
- Commercial exploitation of the developed toxicological testing methods and assessment approaches, products and services;
- Advancement of international co-operation in the field of predictive toxicology and human safety testing;
- Reduced use of laboratory animals in safety testing.

The date for the submission of proposals was 24 February 2015. There are obvious relationships between the objectives, the expected impacts listed above and the outcomes of the **SEURAT-1** Research Initiative. Therefore, the successful consortium was contacted by COACH (see section 4.8) as soon as the negotiation phase with the European Commission was successfully completed.

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, FP7, 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/

Potential for 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 was active for four years. ChemScreen was a sister project of the US Environmental Protection Agency's (EPA) National Center for Computational Toxicology (NCCT/STAR centre). It was 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 worked 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 ended in September 2014. The main objective of the diXa project was 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 focused 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 equips the toxicogenomics community with clear communication channels and agreed core service support. Contributions include 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 demonstrated the feasibility of its approach by performing cross-platform integrative statistical analyses and cross-study meta-analyses to create a systems model for predicting chemical-induced liver injury.

Scientific Coordinator: Jos Kleinjans (University of Maastricht, The Netherlands)

More information: http://www.dixa-fp7.eu/

Existing collaboration: Clemens Wittwehr from the **SEURAT-1** consortium (Joint Research Centre, Ispra, Italy) is a partner in the diXa project.

IMI: Innovative Medicines Initiative

The Innovative Medicines Initiative Joint Undertaking (IMI JU) is a 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. The IMI research

projects that are selected for funding through open calls for proposals must adhere to the four major axes of research defined in the strategic research agenda for the second funding period (IMI 2) from 2014 – 2024: (i) target validation and biomarker research (efficacy and safety); (ii) adoption of innovative clinical trial paradigms; (iii) innovative medicines; and (iv) patient-tailored adherence programmes. In total, four calls for proposals were launched in 2014 (IMI 2 Call 1 – Call 4). The goals of IMI 2 are to develop next generation vaccines, medicines and treatments. The most relevant IMI projects for the **SEURAT-1** activities are briefly described below.

More information: http://www.imi.europa.eu/

EBiSC (*European Bank for induced pluripotent Stem Cells*): EBiSC is funded by the Innovative Medicines Initiative Joint Undertaking (IMI JU). The project started in 2014 and will run until end of 2016. 25 partner institutions are involved in EBiSC. The overall goal of the project is to establish a European iPS cell bank that will be the 'go-to' resource for the characterisation, storage and distribution of high quality iPS cells. Ultimately, EBiSC will become an independent organisation, distributing high quality iPS cells to scientists worldwide on a not-for-profit basis. The mission is that academic institutions, biotech companies, and large pharmaceutical companies can store and access high-quality, well-characterised iPS cells covering a range of disease areas as well as cells from healthy donors. The bank will provide standardised protocols for the storage, retrieval, culture, and differentiation into different cells types, plus a searchable catalogue where cells can be requested based on specific characteristics or disease areas. Samples of the cell lines in the catalogue will be shipped to scientists around the world.

The project partners themselves will generate and deposit the first lines to the bank, but the project will also receive deposits from other projects and organisations. Once the initial collection of cell lines is in place, the project will quickly start distributing cells to the scientific community. The project will then scale up its activities to become fully operational by 2016, having in excess of a thousand lines in its catalogue. The objective is for the initiative to be self-sustaining financially by 2019 and become an independent legal entity, distributing iPS cells worldwide on a not-for-profit basis.

Scientific Coordinator: Timothy Allsopp (Pfizer Ltd, Basel, UK)

More information: http://www.ebisc.org/

Existing collaboration: The **SEURAT-1** Stem Cell Working Group, comprised of researchers from the *SCR&Tox* and ToxBank projects, share the goal of offering well-characterised cell-lines and standard operating procedures. There is an overlap between the EBiSC consortium and **SEURAT-1** projects ensuring exchange activities.

StemBANCC *(Stem cells for biological assays of novel drugs and predictive toxicology):* The IMI project StemBANCC started in 2012 and will run for five 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 bio-repository of well-characterised human iPSCs from different disease groups. Key components in the work programme include: (i) the provision of biomaterials and bio-data; (ii) cellular phenotypic discovery; and (iii) assay development and validation.

Project Coordinator: Martin Graf (F. Hoffmann-La Roche Ltd, Basel, Switzerland)

More information: http://stembancc.org/

Potential for 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. With respect to 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 the use of 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. Exchange activities were initiated through the invitation of the MIP-DILI coordinator, Kevin Park, to the **SEURAT-1** workshop 'Mechanisms underlying repeated dose systemic toxicity' held in November 2011 in Ispra and culminated in a telephone conference between **SEURAT-1** and MIP-DILI partners in December 2014, in which possible areas of collaboration were further explored (see section 5.3).

eTOX *(Integrating bioinformatics and chemoinformatics approaches for the development of expert systems allowing the in silico prediction of toxicities):* eTOX (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 is 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 was terminated in June 2015. Overall, 20 partner organisations worked together to improve the drug development process through the development of tools for the 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 was to identify, a set of biomarkers for each of the three organ toxicities 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 were 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/

Potential for cooperation: SAFE-T worked 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. £4 million was 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) under the umbrella of the 'Advancing the development and application of non-animal technologies' competition. A key aim of the funding is to harness the commercial potential of technologies in this area, including fields related to biological-, tissue engineering and imaging (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 of new chemicals.

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 at the end of March 2014. Fifteen business collaborations, carrying out early-stage investigations into the feasibility of novel non-animal technologies, were selected for funding in July 2014.

More information: http://www.nc3rs.org.uk/

Potential for cooperation: Such feasibility studies as outlined above are highly relevant to the **SEURAT-1** Research Initiative.

CEFIC *(The European Chemical Industry Council)*: CEFIC represents 29,000 large, medium and small chemical companies in Europe and it is the forum and 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 where scientific knowledge relevant for both the industry and regulators should be enhanced were identified. Funding is being made available for research in these areas through requests for proposals. The most relevant requests for proposals to the **SEURAT-1** Research Initiative in 2014 was entitled 'Mining data (bases) to expand the domain of applicability of chemical activity' (code: LRI-ECO30). The objectives of this research programme are (i) to expand the domain of applicability of chemical activity using existing data sets, including but not limited to other non-baseline toxicity modes-of-action, endpoints, and *in vitro* data; (ii) to explore approaches /strategies for utilising acute and chronic data of lesser quality in the absence of high quality data; and (iii) to determine if MoA and chemical activities cluster in large *in vitro* data sets (e.g. USEPA ToxCast). The deadline for proposal submissions was 31 August 2014 and the project is expected to run for up to two years.

CEFIC is currently drafting proposals on how workplace regulation, including occupational exposure limits, can 'interact' with REACH processes such as authorisation. It is implementing this change because although REACH says uses of substances subject to authorisation may be exempted if the health risks are controlled by minimum requirements, in practice this has never happened in practice.

More information: http://www.cefic-lri.org/

Potential for collaboration: Data mining strategies as outlined above are central to determining modes-of-action relevant for the development and refinement of AOPs. Furthermore, these strategies are being used within the **SEURAT-1** Research Initiative in the context of category formation (COSMOS) and read-across.

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 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 to promote 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 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** projects COSMOS and the JRC are actively collaborating with the OECD in developing the AOP framework. The prototype AOPs developed and investigated within **SEURAT-1** as a result of work within the **SEURAT-1** projects DETECTIVE and *HeMiBio* 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 organisations within 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: Russell Thomas (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/Tox21/

Existing cooperation: A joint meeting between **SEURAT-1** and Tox21 occurred in June 2013 in Ispra, Italy. Common interests as a basis for future collaboration were discussed on this occasion. In the meantime, exchange activities were 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 involved the profiling of approximately 1800 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, food additives, 'green' chemicals, industrial chemicals and consumer products, and nanomaterials were 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: Russell Thomas (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/toxcast/

Existing 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 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 the mutual use of repeated dose toxicity data in the respective data bases. Further collaborations are envisaged (see entry above under Tox21).

The Virtual Tissues Research Project: The Virtual Tissues Research 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 that simulates dynamic biological processes.

Since the liver frequently shows the earliest signs of injury, the Virtual Liver (v-Liver™) project is researching how to simulate liver function that can be used to help predict the effects of chemicals in humans. v-Liver plans to use fast, automated chemical screening data from ToxCast™ and other chemical data to simulate how chemicals could cause liver toxicity. Other computer-simulated models being developed are the Virtual Embryo (v-Embryo™) models, which will provide insights into how pregnant mother's exposures to chemicals in the environment might affect prenatal development.

The mechanistic understanding of chemical effect networks will serve as the basis for modelling the key molecular, cellular and circulatory systems in both application areas. The Virtual Tissues team includes an interdisciplinary team of toxicologists, computer engineers, programmers, bioinformaticians, biologists, mathematicians, and other experts. The team aims to use a selection of everyday chemicals with known health effects in animals to develop methods to use vast collections of data, biological knowledge-bases and high-tech computer modelling to build computer-based virtual models.

More information:
http://www.epa.gov/ncct/download_files/factsheets/virtual_tissues_ research_project.pdf

Other components of the 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 Interactive Chemical Safety for Sustainability (**iCSS**) dashboard provides an interactive tool to explore rapid, automated (or *in vitro* high-throughput) chemical screening data generated by the ToxCast™ project (see above) or other components of the Tox21 collaboration.

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/

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. DrugMatrix contains a graphic user interface for rapid scoring of genomic signatures of toxicity. The database 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

Existing 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 are an important resource for identifying key events within AOPs.

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. The ultimate goal is to combine all major organ systems to form a so-called human-body-on-a-chip.

In the first funding phase (2012-2014), the NIH issued 19 awards, 11 of which supported studies to develop 3D cellular microsystems that represent a number of human organ systems. The additional seven awards explored 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.

The goal for the second funding phase (2014-2017) is to further refine the technology and begin organ-chip integration by means of renewable cell sources and bioengineered microsystems that successfully demonstrated physiological function in the first phase.

More information: http://www.ncats.nih.gov/tissuechip/about

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 the 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) develop plans to improve communications between stakeholders and the public (e.g. through focused workshops); and (iii) 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 18 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/

ILSI / HESI *(International Life Sciences Institute / Health and Environmental Sciences Institute)*: ILSI is a non-profit, worldwide organisation whose mission is to provide science that improves human health and well-being and safeguards the environment. ILSI is located in Washington D.C., USA. HESI was established in 1989 as a global branch of ILSI. The intention of ILSI / HESI 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. It 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.

HESI created the RISK21 project, which developed a framework for integrating animal-free testing methods into human health risk assessment. An overview of this framework and the RISK21 project is given in section 2.4 of this Annual Report.

Executive Director: Syril Pettit (Health and Environmental Science Institute, Washington D.C., USA)

More information: http://www.ilsi.org/; http://www.hesiglobal.org/

Existing cooperation: The **SEURAT-1** project COSMOS has established two expert groups with ILSI Europe: 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.

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) and the **SEURAT-1** case studies make use of this important resource.

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 2014 annual conference was entitled 'How to facilitate the use of alternative methods by regulators?' and was held in Brussels on 19 November 2014. 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/growth/sectors/chemicals/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/

EUSAAT 2015: 16th Annual Congress of the European Society for Alternatives to Animal Testing

Date: 20–23 September 2015

Location: Linz, Austria

The goals of the European Society for Alternatives to Animal Testing (EUSAAT) are to support: (i) the dissemination and validation of alternative methods to animal testing; (ii) the promotion of research in the field of the 3Rs (refine, reduce, replace); (iii) the reduction of the use of animals for tests in the field of education and continuing education; (iv) the reduction of sufferance and stress of laboratory animals by better breeding, keeping, test planning and other accompanying measures; (v) the provision of specialist guidance and expert opinions for public and private organisations, companies and universities; (vi) suitable information for the public and the media.

Main themes of the congress for discussion are:

- New Technologies: Systems Biology, '-omics' Technologies, 3D Models;
- International Progress in 3Rs Research and Global Cooperation on Implementing the 3Rs;
- Replacement – New Approaches;
- Predictive Toxicology (with a focus on QSAR, Read Across and the AOP Concept);
- Specific Toxicological Endpoints (including Repeated-Dose Toxicity);
- Efficacy and Safety Testing of Drugs, Biological and Vaccines;

- Disease Models *in vivo* and *in vitro*;
- Refinement and Welfare, Culture of Care, Best Practice Approaches, Avoidance of Severe Suffering;
- 3Rs in Academia and Education;
- Ethical and Legal Issues.

More information: http://www.eusaat.org/

4th Annual Meeting of the American Society for Cellular and Computational Toxicology (ASCCT)

Date: 1–2 October 2015

Location: Durham, North Carolina, USA

The ASCCT is a scientific society dedicated to the promotion of toxicology testing and research that reduces and replaces the use of animals. The 'Physicians Committee for Responsible Medicine' and the 'Institute for *In Vitro* Sciences, formed the ASCCT in 2010 to foster cooperation and dialogue among North American scientists, regulators and non-governmental organisations from the pharmaceutical, chemical, pesticide and consumer product sectors.

The ASCCT will hold their annual meeting, entitled 'Integrated Approaches to Testing and Assessment: Promises and Challenges of a More Flexible Approach to Toxicology Testing, in October. The main session topics for the ASCCT 2015 meeting are:

- IATA Application Case Studies;
- Chemicals and Risk: New Approaches to Current Practices.

More information: http://www.ascctox.org/meetings.cfm

IVTS: In Vitro Toxicology Society

Date: 10–11 November 2015

Location: Birmingham, UK

The IVTS was founded under a constitution in 1988 for scientists active in the study, practice or development of *in vitro* toxicology. The aims of the IVTS are (i) to encourage alternative approaches to animal testing and promoting the 3Rs; (ii) to provide a forum for discussion for scientists actively involved in the study, practice or development of *in vitro* toxicology; (iii) to arrange scientific meetings on the subject of *in vitro* toxicology and its practical applications;

(iv) to promote an exacting scientific approach to the practice of *in vitro* toxicology; and (v) to encourage participation of new student scientists in the field of *in vitro* toxicology.

The IVTS will host their annual meeting in November, with topics ranging from regulatory issues through to innovative *in vitro* models, providing an excellent forum for sharing information and cross-sector and cross-disciplinary collaboration. The main session topics for the IVTS 2015 meeting are:

- Hepatoxicity *in vitro*;
- Inhalation Toxicology *in vitro*;
- Developmental and Reproductive Toxicology *in vitro*;
- Neurotoxicology *in vitro*.

More information: http://www.ivts.org.uk/site/ivts2015/

CCT: Contemporary Concepts in Toxicology Meetings. FutureTox III: Bridges for Translation

Date: 19–20 November 2015

Location: Arlington, Virginia, USA

The Society of Toxicology (SOT) conducts Contemporary Concepts in Toxicology (CCT) Meetings to achieve the SOT Strategic Objective of providing tools and resources to members that will enhance their professional and scientific development, as well as to provide an opportunity for building improved understanding of and dialogue around emerging science critical to advancing the practice of toxicology.

An upcoming CCT Meeting is entitled 'FutureTox III: Transforming 21st Century Science into Risk Assessment and Regulatory Decision-Making'. The first FutureTox meeting in 2012 focused on challenges and opportunities associated with implementing 21st century toxicity testing technologies. In 2014, FutureTox II focused on the science to advance an outcome paradigm where improvements to predictivity and concordance are based solely on *in vitro/ in silico* approaches. FutureTox III will focus on building the high-throughput risk assessment paradigm, taking the science of *in vitro* data and *in silico* models forward.

Main themes of the meeting for discussion are:

- Hazard Characterisation Using Tox21 Tools/Approaches;
- Exposure Characterisation Using Tox21 Tools/Approaches;
- 21st Century Risk Assessment – How New Approaches Can Impact Regulatory-Decision Making.

More information: http://www.toxicology.org/events/shm/cct/futureToxIII.asp

HeMiBio International Symposium: Biology Meets Technology for Liver Toxicity Testing

Date: 2–3 December 2015

Location: Leuven, Belgium

The symposium will highlight the results obtained within the *HeMiBio* consortium, including the development and differentiation of iPSC reporter lines and hepatic differentiation protocols, microfluidic bioreactor design and construction and development of sensors capable of evaluating hepatocyte function in repeated dose toxicity settings; and the creation of an *in vitro* model hepatocyte-stellate cell co-culture model for assessment of liver fibrosis. In addition, sessions will also highlight topics covered by other **SEURAT-1** funded consortia, including state-of-the-art lectures by experts in these areas of research. Sessions will focus on:

- Development of Liver Bioreactors for Drug Development and Therapeutics;
- Combining Pluripotent stem cell Differentiation and Genome Engineering to Create Liver Tissue *in vitro*;
- Implementation of the Adverse Outcome Pathway concept in *HeMiBio*.

More information: http://www.hemibio.eu/events/events-details

SEURAT-1 Symposium: Painting the Future Animal-Free Safety Assessment of Chemical Substances: Achievements of SEURAT-1

Date: 4 December 2015

Location: Brussels, Belgium

The symposium is a great opportunity to learn about the recent achievements in the field of alternative testing strategies, network with renowned experts and get acquainted with the activities of other on-going and future initiatives.

High-level presentations will first showcase the **SEURAT-1** success stories in a practical and accessible manner, an open exhibition with demonstrations organised at dedicated information booths will allow for deeper discussions. The attendees will learn how the extensive research efforts during the last 5 years can be translated into solutions for safety assessment ultimately replacing animal testing. Other related on-going and future initiatives from the EU and US will be invited to showcase their progress in the field of alternative testing strategies and thus stimulate exchange and networking.

More information: http://www.seurat-1.eu/pages/library/events/seurat-1-symposium.php

Systems Toxicology 2016: Real World Applications and Opportunities

Date: 27–29 January 2016

Location: Les Diablerets, Switzerland

Building on the highly successful 2013 Systems Toxicology conference in Ascona, the 2016 meeting explores current and likely future specific applications of systems toxicology approaches in chemical risk evaluation.

Main themes of the conference for discussion are:

- From Exposure To Population Impact;
- Reliable Model Systems To Measure Key Events;
- Integrative Analysis Of '-Omics' Measurements In Systems Toxicology;
- Linking Network Perturbations To Phenotypes;
- Emerging Applications For Systems Toxicology.

More information: http://systox2016.ch/Home/

OTHERS IN THE FIELD

51st Congress of the European Societies of Toxicology

Date: 13–16 September 2015

Location: Porto, Portugal

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 aim of the 2015 conference is 'Bridging Sciences for Safety'.

Main themes for discussion include:

- Regulatory Toxicology;
- Human and Environmental Risk Assessment;
- *in vitro* Models;
- Computational Toxicology;
- '-Omics' Technologies;
- Mechanisms of Toxicity;
- Organ Toxicities.

The **SEURAT-1** Research Initiative is present in several sessions and this Annual Report was launched on this event.

More information: http://www.eurotox2015.com/

55th Annual Meeting of the Society of Toxicology (SOT)

Date: 13–17 March 2016

Location: New Orleans, 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: https://www.toxicology.org/events/am/AM2016/

52nd Congress of the European Societies of Toxicology

Date: 4–7 September 2016

Location: Istanbul, Turkey

The motto of the EUROTOX 2015 is 'Protecting Public and Environmental Health by Understanding and Communicating Toxicology'. In addition to presenting cutting-edge research, the EUROTOX annual meetings offer an unparalleled venue for renewing professional relationships, networking and remaining up to date in our challenging and expanding discipline.

More information: http://www.eurotox2016.com/

5.3 SEURAT-1 Meets Mip-DILI

Elisabet Berggren

An ambition of the **SEURAT-1** Research Initiative is to connect with other scientific projects and initiatives focused on the development of alternative methods to traditional animal testing as a basis for safety assessment. In this spirit, collaboration with the American Tox21 and ToxCast projects as well as other **SEURAT-1** partners, was established in 2013. In December 2014 a preparatory telephone conference was set up between **SEURAT-1** and MIP-DILI partners to further explore possible areas of collaboration or knowledge exchange.

MIP-DILI (Mechanism-Based Integrated Systems for the Prediction of Drug Induced Liver Injury) is consortium funded by IMI (Innovative Medicines Initiative) with 26 participants from the pharmaceutical industry, SMEs and academic institutions.

The main focus of the discussion was chemical selection for testing *in vitro* systems developed for hepatotoxicity, and the selection criteria for the **SEURAT-1** standard reference compounds were presented. In addition, a common concern was achieving standardisation of methods already available rather than developing new systems, and establishing a common basis for characterisation of current systems. The discussion will continue in 2015 and could be connected with any follow-up project of the **SEURAT-1** Research Initiative.

5.4 Recommendations for Future Research in the Field of Predictive Toxicology

George Daston, Derek Knight, Michael Schwarz, Tilman Gocht, Russell S. Thomas, Catherine Mahony, Maurice Whelan

5.4.1 Background

As the **SEURAT-1** Research Initiative comes towards an end, the planning for a possible follow-up initiative becomes an important task. The work programme for such a follow-up initiative should take the research strategy that was developed as an integrated part of **SEURAT-1** into consideration and develop it further based on the achievements made and gaps identified in **SEURAT-1**. However, a pure continuation of the **SEURAT-1** work programme is not intended, but it seems reasonable to define the scope and aims for the next **SEURAT** phase upfront. Therefore, a planning group consisting of the authors of this section was established with the following tasks:

- To elaborate the scope and aims of the follow up initiative based on the vision and strategy defined in **SEURAT-1**
- To define an outline research programme and the components to reach the aims.

The outcomes of the work from the planning group were published (*Daston et al., 2015*) and in the following we present an extended summary of the article published in *Archives of Toxicology*. The considerations made by the planning group were motivated by **SEURAT-1**, but are not restricted to a follow-up research programme and may be relevant for any consortia that will be formed based on current or future calls for proposals in the field of predictive toxicity.

5.4.2 Recommendations for Next Steps

Drawing on the experience gained in **SEURAT-1** and appreciating international advancement in both basic and regulatory science, we reflect here on how SEURAT should evolve scientifically to ultimately realise its vision. Essentially we propose that further research and development should be directed along two complementary and interconnecting work streams (*Figure 5.1*).

The first work stream would focus on developing new ‘paradigm’ approaches for regulatory science. The goal here is the identification of ‘critical biological targets’ relevant for toxicity

and to test their suitability to be used as anchors for predicting toxicity. These critical targets will not be restricted to effects on distinct organs or particular types of toxic effects. Rather, the idea is that, independent of the nature of the adverse outcome of interest, be it cancer, developmental toxicity or acute or repeated dose toxicity, the disturbance of pathways related with these critical biological targets would indicate a likelihood for adversity and the dose at which this would occur. The aim would be to establish, at a proof-of-concept level, the suitability of this 'critical target concept' to improve the predictive power of mechanism-based toxicity testing methods, which might be applied in a future regulatory safety assessment. The central aspect of this work stream is to give up the definition of adversity at the organ level and to identify new points-of-departure for a future safety assessment paradigm at the molecular scale.

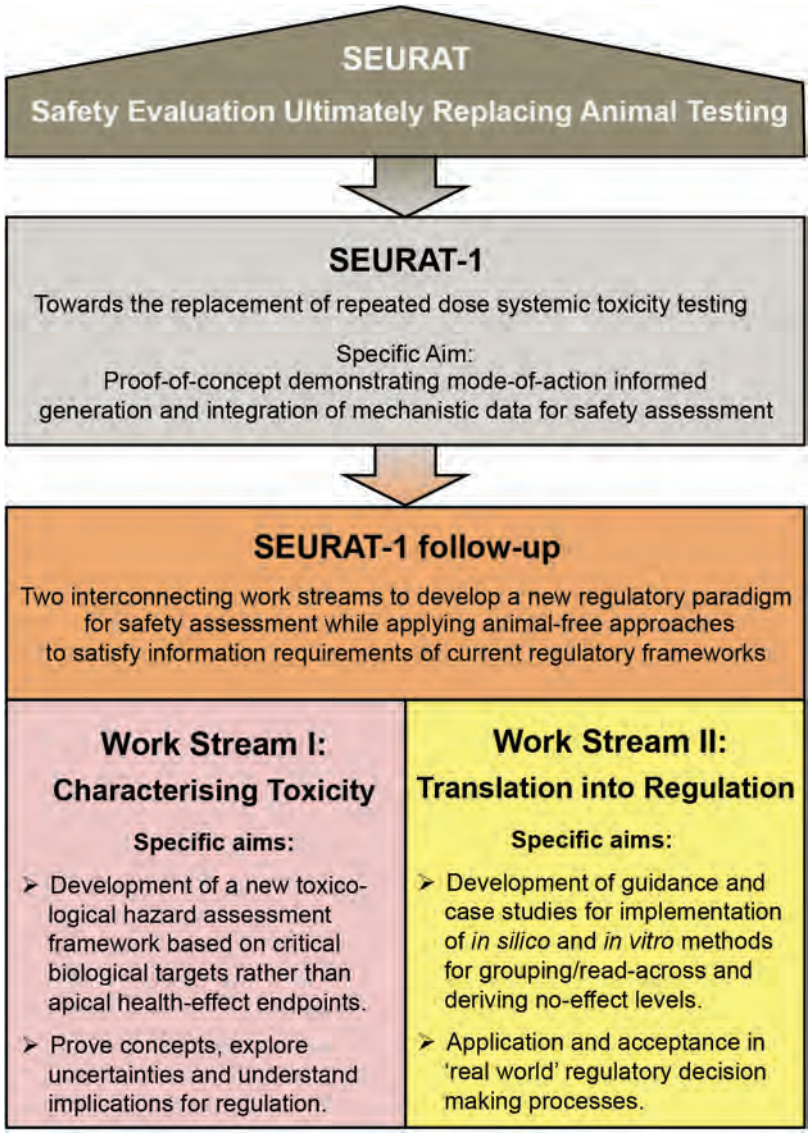

Figure 5.1 *Realising the SEURAT vision - A two-pronged approach to advance predictive toxicology and safety assessment to assure the highest levels of protection for human health while avoiding animal testing (source: Daston et al., 2015).*

The second work stream would focus on integration and application of new approach methods for hazard (and risk) assessment within the current regulatory 'paradigm', aiming for acceptance of animal-free testing strategies by regulatory authorities (i.e., translating scientific achievements into regulation). In essence, this would be to complete the **SEURAT-1**

'conceptual framework' (see section 4.10.9) for combining evidence, in particular from new-approach methods, in a biologically-rational manner to qualitatively and quantitatively predict traditional organ-based toxicity. Specific implementation of the framework includes contributing additional evidence of the biological basis for 'read-across' and *ab initio* development of a safety assessment that relies only on the new *in silico* and *in vitro* methods. The aim is to develop guidance (e.g., at OECD level) for the implementation of non-standard *in silico* and *in vitro* methods into regulatory risk assessment. The application in 'real-world' decision-making processes is an essential deliverable. In this way, this work stream would be a continuation of the **SEURAT-1** programme, which would evolve beyond the proof-of-concept stage and finalise the work started in **SEURAT-1**, demonstrating the widespread implementation of mode-of-action based reasoning in chemical safety assessment and regulatory decision making.

The conceptual underpinning of these two work streams builds on the current state-of-the-art in different research areas that will need to be developed and/or perfected to deliver the components needed for these work streams. These include chemoinformatics, high throughput screening and high-content methods (e.g., toxicogenomics), systems biology approaches (both computational and lab-based (tissue chip) models), pharmacokinetic modeling (including reverse- dosimetry and QVIVE) and the refinement of safety assessment frameworks. Further details are given in *Daston et al. (2015)*.

DISCLAIMER

The views expressed in this article are those of the authors and do not necessarily reflect the views or policies of the US Environmental Protection Agency, the European Commission and the European Chemicals Agency.

Reference

Daston, G., Knight, D.J., Schwarz, M., Gocht, T., Thomas, R.S., Mahony, C., Whelan, M. (2015): SEURAT: Safety Evaluation Ultimately Replacing Animal Testing – Recommendations for future research in the field of predictive toxicology.- *Arch. Toxicol.*, 89: 15-23.

Glossary

This glossary was compiled from various existing reference documents coming from different sources. When possible, we used the *Glossary of Reference Terms for Alternative Test Methods and their Validation* published by *Ferrario et al.* (*2014*) in ALTEX (Vol. 31, Is. 3, p. 319-335) as a point of reference (an internet-based version of this published compilation can be found at: http://altweb.jhsph.edu/resources/validation_glossary.html).

3Rs Reduction, replacement, refinement - defined by Russel & Birch 1959. Reduction is any means of lowering the number of animals used to obtain information of a given amount and precision. Refinement is any development that refines procedures to lessen or eliminate pain or distress to animals, or enhances animal well-being. Replacement is any scientific method employing non-sentient material, which may replace methods which use conscious living vertebrates.

AC Activity concentration (AC10 / AC50 = activity concentration at 10% / 50% of response)

ADME Absorption, Distribution, Metabolism, and Elimination. ADME describes the disposition of a pharmaceutical compound within an organism (see also TK, toxicokinetics).

ADMET Absorption, Distribution, Metabolism, Excretion, and Toxicokinetics 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 AOP is a sequence of events from the exposure of an individual or population to a chemical substance through a final adverse (toxic) effect at the individual level (for human health) or population level (for ecotoxicological endpoints). The key events in an AOP should be definable and make sense from a physiological and biochemical perspective. AOPs incorporate the toxicity pathway and mode of action for an adverse effect. AOPs may be related to other mechanisms and pathways as well as to detoxification routes.

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. In terms of statistics, the BMD is the calculated lower 95% confidence limit on the dose that produces a defined response (called the benchmark response or BMR, usually 5% or 10%) of an adverse effect compared to background, often defined as 0% or 5%.

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

DPRA Direct Peptide Reactivity Assay

EB Embryoid body

EC Endothelial cell

EC SCCS European Commission Scientific Committee on Consumer Safety (see entry under 'SCCS')

EC_{50} (median effective concentration): Statistically derived median concentration of a substance in an environmental medium expected to produce a certain effect in 50% of test organisms in a given population under a defined set of conditions.

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: A set of principles that provide a framework within which laboratory studies are planned, performed, monitored, recorded, reported, and archived. GLP helps to assure regulatory authorities that the data submitted are a true reflection of the results obtained during the study, and can therefore be relied upon when making risk/safety assessments.

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

hitc Hit-call. Parameter used to determine the activity for the concentration series ind a dose-response curve.

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: The use of robotics-based technology to screen large sets of substances for specific activities.

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

ISA-TAB Investigation-Study-Assay TAB delimited format. The ISAcreator software is used to create archives containing experiment descriptions as well as the raw data of an investigation. An archive typically includes all the work that is part of a publication. The archive contains three tables describing the experimental set-up in a hierarchical fashion. The upper level table in the ISA-TAB archive is known as an Investigation. An Investigation contains one or more Studies. Studies share the use of similar biological materials, e.g. same types of treatments and cells that are investigated for instance using different technologies. A Study contains one or more Assays. Each assay is technology-specific and common features associated with a particular technology (e.g. affymetrix microarrays) are captured in **SEURAT-1**/ToxBank assay templates. An Assay contains links to one or more data files. The table contains links to these data files and details about the protocols that were used to derive them.

iTRAQ Isobaric Tag for Relative and Absolute Quantitation

ITS Integrated Testing Strategy. In the context of safety assessment, an integrated testing

strategy is a methodology which integrates information for toxicological evaluation from more than one source, thus facilitating decision-making. This should be achieved whilst taking into consideration the principles of the 3 R's (reduction, refinement, and replacement).

IVIVE *In Vitro* Concentration to *In Vivo* Dose Extrapolation

JNK c-Jun NH(2)-terminal protein kinase pathway

KE Key Eevents: Steps along the pathway that represent intermediate events, typically at the different levels of biological organisation which are experimentally or toxicologically associated with an adverse outcome pathway.

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.

LLNA Local Lymph Node Assay. This assay is a murine model developed to evaluate the skin sensitization potential of chemicals.

LOAEL Lowest Observed Adverse Effect Level: Lowest concentration or amount of a substance (dose), found by experiment or observation, which causes an adverse effect on morphology, functional capacity, growth, development, or lifespan of a target organism distinguishable from normal (control) organisms of the same species and strain under defined conditions of exposure.

LOEL Lowest Observed Effect Level: Lowest concentration or amount of a substance (dose), found by experiment or observation, that causes any alteration in morphology, functional capacity, growth, development, or lifespan of target organisms distinguishable from normal (control) organisms of the same species and strain under the same defined conditions of exposure.

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. It is the specific biochemical interactions through which a substance produces its effect. Mechanism of action refers to a detailed description, often at molecular level, of the means by which an agent causes a disease state or other adverse effect.

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 starts the pathway 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 (see NOAEL).

NOAEL No observed adverse effect level: Greatest concentration or amount of a substance, found by experiment or observation, which causes no detectable adverse alteration of morphology, functional capacity, growth, development, or lifespan of the target organism under defined conditions of exposure.

NOEL No observed effect level: Greatest concentration or amount of a substance, found by experiment or observation, that causes no alterations of morphology, functional capacity, growth, development, or lifespan of target organisms distinguishable from those observed in normal (control) organisms of the same species and strain under the same defined conditions of exposure.

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

Pharmacokinetics Process of the uptake of drugs by the body, the biotransformation they undergo, the distribution of the drugs and their metabolites in the tissues, and the elimination of the drugs and their metabolites from the body.

PHCP Personal and household care products

PHH Primary Human Hepatocytes: Primary cells that are freshly isolated from human sources (the liver in case of hepatocytes). Freshly isolated primary cells may rapidly dedifferentiate in

culture, and they have a limited lifespan. Primary cell cultures commonly require complex nutrient media, supplemented with serum and other components. Consequently, primary cell culture systems are extremely difficult to standardise.

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 Quantitative Structure-Activity Relationship: A QSAR is a theoretical model for making predictions of physicochemical properties, environmental fate parameters, or biological effects (including toxic effects in environmental and mammalian species). QSARs relate quantitative measures of chemical structure to continuous or categorical variables describing the property to be predicted.

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. The European Community Regulation on chemicals and their safe use (EC 1907/2006). The new law entered into force on June 1, 2007. The aim of REACH is to improve the protection of human health and the environment through the better and earlier identification of the intrinsic

properties of chemical substances. At the same time, innovative capability and competitiveness of the EU chemicals industry should be enhanced.

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: A theoretical model for making predictions of physicochemical properties, environmental fate parameters, or biological effects (including toxic effects in environmental and mammalian species). SARs are qualitative relationships in the form of structural alerts that incorporate molecular substructures or fragments related to the presence or absence of activity.

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: A formal, written procedure that describes in detail how specific routine and test-specific laboratory operations should be performed. SOPs are required by Good Laboratory Practice.

SQL Often referred to as 'Structured Query Language' is a programming language designed for data management.

SREBP-1c: Sterol Regulatory Element-Binding Protein 1c

Structural alerts Atom-based fragments which, when present in a molecule, are an indication that a compound can be placed into a particular category.

STTF SEURAT-1 Training Task Force

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: Process of interaction of potentially toxic substances with target sites, and the biochemical and physiological consequences leading to adverse effects.

TG-Gates Data-base of the Japanese Toxicogenomics project - Genomics assisted toxicity evaluation system (http://toxico.nibio.go.jp/english/index.html).

TK Toxicokinetics: Generally, the overall process of the absorption (uptake) of potentially toxic substances by the body, the distribution of the substances and their metabolites in tissues and organs, their metabolism (biotransformation), and the elimination of the substances and their metabolites from the body. In validating a toxicological study, the collection of toxicokinetic data, either as an integral component in the conduct of non-clinical toxicity studies or in specially designed supportive studies, in order to assess systemic exposure.

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 Cellular response pathways that, when sufficiently perturbed, are 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: Human exposure threshold value for a group of chemicals below which there should be no appreciable risk to human health. 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

VPA Valproic acid

Web Service A method of communication between two electronic devices over a network.

WoE Weight of Evidence: The process of considering the strengths and weaknesses of various pieces of information in reaching and supporting a conclusion concerning the hazard potential of a substance.

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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* A detailed description about the role of the Scientific Expert Panel including information about the members can be found in the Introduction

- On 11 March 2013 the full ban on animal testing for cosmetic products came into force. From this date the marketing of new cosmetic products tested on animals in the European Union is prohibited in accordance with the seventh amendment of the 'Council Directive on the approximation of the laws of the Member States relating to cosmetic products' (7677687EEC9). The European Commission, together with Cosmetics Europe, launched the Research Initiative 'Towards the replacement of *in vivo* repeated dose systemic toxicity testing' in order to develop a sound research strategy leading to the long-term target of 'Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT). This Research Initiative is called **SEURAT-1** and comprises six research projects focusing on the development of new test methods in the field of repeated dose systemic toxicity.

- This is the fifth 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.