Towards the Replacement of in vivo Repeated Dose Systemic Toxicity Testing

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

Volume 2 2012
« 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.

Paracelsus (1493 - 1541)
Portrait by Quentin Massys

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

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

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

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“Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals”
The consolidation of the Research Strategy

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

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

Safety Evaluation Ultimately Replacing Animal Testing” (SEURAT) is recognised worldwide as a challenging long-term target in predictive human safety assessment and in the related fields of research, technological development, and innovation. Originally triggered and driven by legislation in the field of animal welfare (e.g. Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes), today the main driving forces are human and environmental safety. Animal welfare and issues related to the 3Rs are included as overarching principles in all relevant EU policies.

Science aimed at the mechanistic understanding and prediction of toxic effects on living organisms caused by chemicals has made enormous progress. Predictive toxicology without the use of animals is needed for the development and marketing of many innovative products ranging from new drugs and cosmetic products to industrial chemicals, pesticides, food additives, and ‘biologicals’. The number of toxicological endpoints to be considered is high and the interrelation and interactions of chemicals add to the complexity of the issue.

Scientifically, the area of safety assessment is extremely complex. Hence, the replacement of animal tests is very difficult, in particular in the case of long-term, chronic exposure of the organism to chemicals. A first step has been made with the SEURAT-1 Research Initiative, which started in 2011 and focuses on “Towards the replacement of in vivo repeated dose systemic toxicity testing”. The aim is to construct a number of important functional ‘building blocks’ as a basis for the development of a research strategy to be implemented in the years to follow.

It is clear that the development and implementation of the SEURAT-1 research strategy requires more ‘ingredients’ in addition to the six ‘building blocks’ constructed by the six projects in the SEURAT-1 Research Initiative. Detailed gap analyses are needed as well as excellent monitoring and communication of with all relevant national and international RTD&I activities, followed by a continuous adjustment of the work at cluster level. The coordination of the cluster of projects and the respective communication activities are in the hands of all project teams involved - with the guidance and infrastructure provided by the coordination action COACH and the Scientific Experts Panel (SEP). In the first year of SEURAT-1 the cluster-internal coordination has made good progress and will be intensified through Summer Schools, exchange of scientists, cooperation in specific scientific tasks. In the future – and in addition - more emphasis will be put on international cooperation and on filling the identified knowledge gaps through the creation of new tailor-made projects or other actions.

The complexity of setting up a functioning long-term research initiative is in a certain way comparable with the scientific tasks to be dealt with: understanding of cell signalling, the normal communication that governs basic cellular activities and coordinates cell actions, and the understanding of potential adverse effects chemicals may have on the internal and external communication and functioning of cells, organs, and organisms. In view of the overall target – human safety – it is obvious that the role and application of knowledge originating from computational chemistry and systems biology will have to be increased. Hopefully, and besides the positive effects regarding animal welfare, this will also have a significant impact on the further development and recognition of the emerging field of systems medicine.

One has to keep in mind that this endeavour can only be handled through the intensification of existing and the creation of new partnerships, at national, international, public, and private levels.

We would like to wish all project teams involved every success!

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Executive Summary

This publication is the second volume of a series of six Annual Reports that summarises the activities of a Research Initiative in the field of repeated dose systemic toxicity, which started on 1 January 2011. The Research Initiative is co-funded by the European Commission Directorate-General for Research & Innovation within the HEALTH theme of the Seventh European Research Programme (FP7) and Cosmetics Europe. The framework for this Research Initiative was created in June 2009 through the FP7 call for proposals ‘Alternative Testing Strategies: Towards the replacement of in vivo repeated dose systemic toxicity testing’ with a total funding of EUR 50 million. The Research Initiative follows the long-term target in chemical safety testing ‘Safety Evaluation Ultimately Replacing Animal Testing’ (SEURAT), which was presented by the FP7 HEALTH theme in 2008. 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 SEURAT.

The aim of the SEURAT-1 Research Initiative is the development of a long-term research strategy for research and development work leading to pathway-based human safety assessments in the field of repeated dose systemic toxicity testing of chemicals. The overall goal is to establish animal-free Innovative Toxicity Testing (ITT) methods, enabling robust safety assessment that will be more predictive than existing testing procedures. In order to achieve this, a cluster of projects has been organised, consisting of five research projects, 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 orientation of the SEURAT-1 Research Initiative and, thus, plays a key role in its scientific coordination.
Objectives of the SEURAT-1 Research Initiative

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<td>Develop highly innovative tools and methodology that can ultimately support regulatory safety assessment</td>
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<td>Formulate and implement a research strategy based on generating and applying knowledge of mode-of-action</td>
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<td>Demonstrate proof-of-concept at multiple levels - theoretical, systems, and application</td>
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<td>Provide the blueprint for expanding the applicability domains - chemical, toxicological and regulatory</td>
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The SEURAT-1 Research Initiative combines the expertise in cell culturing for the preparation of stable human cell lines with the establishment of sophisticated experimental systems such as organ simulating devices, and links the experimental work with advanced methods of computational modelling and estimation techniques, taking innovative systems biology approaches into consideration. This requires a coordinated joint effort of the over 70 European universities, public research institutes and private companies that are involved in the SEURAT-1 Research Initiative. As a first step, key contributions from each of the projects addressing the cluster level objectives were identified (see Figure 1). This is the starting point of a strategic review that will be further elaborated by connecting the different key contributions in a purposeful and effective manner. The infrastructure for such a collaborative, interactive task has been established through the organisation of cross-cluster working groups focussing on (i) the selection of standard reference compounds to be used for toxicity testing (Gold Compounds Working Group); (ii) the data exchange between the projects and the standardisation of data analysis (Data Analysis Working Group); (iii) the identification of modes-of-action relevant for repeated dose systemic toxicity (Mode of Action Working Group); (iv) the in vitro to in vivo extrapolation and the calculation of appropriate concentration ranges to be tested in in vitro experiments (Biokinetics Working Group); (v) the standardisation of quality control issues of the cells used by the different partners and projects (Stem Cells Working Group); (vi) bridging the gap between non-animal toxicity testing and the safety assessment decision making needs (Safety Assessment Working Group).
**Figure 1** Overview of project contributions, essential for achieving cluster objectives and triggering cross-cluster interactions.
The core of this second Annual Report, prepared by the coordination and support action project COACH, is formed by a comprehensive overview of the first results obtained in the different projects of the SEURAT-1 Research Initiative. This is given in the context of recent developments in European legislation regarding the regulation of chemicals to improve their safety assessment and related international activities.

Chapter 1 provides a general introduction to the SEURAT-1 Research Initiative. It describes the history of the call for research proposals under FP7, recent developments with respect to additional funding in related research fields, and provides an overview about the cluster level objectives as well as the structure of the SEURAT-1 Research Initiative.

Chapter 2 outlines the context of the SEURAT-1 Research Initiative from various perspectives.

(i) Legislation: There is little doubt that systemic toxicity will account for a considerable proportion of the testing costs and animal use of REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals, Regulation 1907/2006/EC). As a consequence, the use of ‘non-standard data’ finds more and more acceptance by the regulatory agencies. The registrant can ‘adapt’ the standard information requirements under REACH, and use other, non-standard information instead. The respective approaches should address the key parameters of the standard method and the result must be suitable for adequate risk assessment and/or classification under the Classification, Labelling and Packaging Regulation (CLP, Regulation 1272/2008/EC). Furthermore, the Seventh Amendment (Directive 2003/15/EC) to the Cosmetics Directive (Directive 76/768/EEC) foresees a deadline in 2013 for the replacement of animal testing of cosmetic products in the fields of repeated dose toxicity, reproductive toxicity and toxicokinetics. A study by the European Commission on the possible socio-economic impact of the implementation of the ban is expected to be finalised mid 2012 – this report will cover a number of legislative options (including the full implementation of the ban) to illustrate the type and scope of impact. The outcome of this exercise will help the European Commission to prepare a proposal for consideration by the European Parliament and the Council of ministers (this may happen while this SEURAT-1 Annual Report is being printed). (ii) Science: Methods to obtain appropriate non-standard data in the regulatory context comprise in vitro studies, the use of human epidemiology data, information from structurally-related substances (i.e. ‘read-across’ and ‘chemical categories’), predictions from valid (Q)SARs and the use of the weight of evidence approach. The state of the science in the development of such methods is presented and discussed in the context of improved understanding about molecular mechanisms of toxicity through the elucidation of toxicity pathways in view to meet the challenge of toxicity testing in the 21st Century.

Chapter 3 focuses on the development of a long-term research strategy and its implementation within the cluster. As already outlined in the first Annual Report, 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. This has triggered the selection of standard reference chemicals to be used in the research projects, which was made based on the clear association of a molecule with a particular mode-of-action and underlying mechanisms or effects. As the identification and full description of modes-of-action in the field of repeated dose systemic toxicity is still an open task, the SEURAT-1 Research Initiative as a whole will follow a case study approach, i.e. a few well-described cases will be selected and toxic effects will be reproduced by both, experimental approaches to identify appropriate readouts ('biomarkers') and in silico approaches to increase the predictive power of the respective computer models. For this, the Adverse Outcome Pathways (AOP) approach will be adopted as the conceptual and practical tool of choice. The final output of the SEURAT-1 Research Initiative will be to deliver a proof-of-concept showing if and how the developed scientific tools and the know-how can be combined in test systems to create innovative decision support for human safety assessment.

**Proof of concepts at multiple levels**

- **Theoretical** – describe selected Modes of Action / Adverse Outcome Pathways to a sufficient extent so that they can be used as blueprints for system design.

- **Systems** – demonstrate integrated systems for associating a chemical with a Mode of Action / Adverse Outcome Pathway category and for predicting points of departure of a pathway of toxicity.

- **Application** – use the information derived from predictive systems to support safety assessment processes and decisions.

The detailed project descriptions and their first results are given in chapter 4. The SEURAT-1 Research Initiative is designed as a coordinated cluster of five research projects, supported by a 'data handling and servicing project' and a 'coordination and support project'. The tasks of the projects and the highlights presented in this Annual Report are:
Stem cell differentiation for providing human-based organ specific target cells to assay toxicity pathways \textit{in vitro}.

The SCR&Tox report focuses on the development of quality control standards that can be applied in routine pluripotent stem cell-based toxicity testing.

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

\textit{HeMiBio} reports on the generation of the different bioreactor prototypes including the incorporation of high-resolution fluorescent markers into pluripotent stem cells as well as the initial complement of electrochemical sensors.

Identification and investigation of human biomarkers in cellular models for repeated dose \textit{in vitro} testing.

DETECTIVE will deliver functional as well as ‘-omics’ biomarkers for different organs (liver, heart and kidney) and reports about first experiments to evaluate the most appropriate human cellular model system for each organ.

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

Data sets likely to be of use to the COSMOS project, suitable for the development of \textit{in silico} models, have been identified. A non-cancer dataset about Thresholds for Toxicological Concern (TTC) for cosmetic ingredients has been compiled and the applicability of the TTC approach to cosmetic ingredients has been explored. Furthermore, a process-based model able to simulate the dynamics of a chemical compound in cell-based assays has been developed as a basis for \textit{in vitro} – \textit{in vivo} extrapolations.

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

NOTOX describes the establishment of a spheroid cultivation system and its successful application in several cell lines. First toxicity tests have been carried out and ‘-omics’ profiles as well as structural changes were
monitored. Tools of integrative and predictive computational systems biology were applied to integrate information obtained from iterative cycles of model predictions and experimental validations by *in vitro* experiments, in order to eventually predict the possible toxicity of test compounds *in vivo*.

**ToxBank**

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

The ToxBank report focuses on the selection of standard reference compounds (‘gold compounds’) for toxicity testing and the development of a shared cross-cluster database to enable an integrated data analysis.

**COACH**

Cluster level coordinating and support action.

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

Chapter 4 also contains reports about the meetings of each of the specific projects as well as of the SEURAT-1 Research Initiative as a whole. These meetings were conducted to provide input into the annual action plan, as well as to foster collaborations between the projects. The elements for establishing optimal exchanges between the projects (e.g., by setting up the above-mentioned cross-cluster working groups) are discussed as well.

Chapter 5 describes the related international activities. The list of short project descriptions that has been launched in the first Annual Report was updated, with special emphasis on initiatives focussing on repeated dose toxicity in combination with stem cell technologies. Furthermore, the 21st century tools needed for innovative safety assessment are briefly introduced and discussed by Melvin Anderson and co-workers, who are following in the USA similar approaches as SEURAT-1. For SEURAT-1 to be successful, it is important to join with the various complementary international research programmes on the way ‘towards the replacement of *in vivo* repeated dose systemic toxicity testing’ and to achieve better human safety assessment of chemicals in the future.
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“A journey of a thousand miles must begin with a single step.”
Lao-Tzu
Background

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’) foresees a deadline in 2013 for the replacement of animal testing of cosmetic products in the fields of repeated dose toxicity, reproductive toxicity and toxicokinetics. Triggered by this deadline, Cosmetics Europe – The Personal Care Association (previously named Colipa) had proposed a contribution of EUR 25 million in the beginning of 2008 to support the research work in one of these most challenging areas in toxicology, which is repeated dose systemic toxicity.

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

In June 2009 the framework for the SEURAT-1 Research Initiative was created through a call for proposals under the HEALTH Theme of the 7th European RTD Framework Programme: ‘Alternative Testing Strategies: Towards the replacement of in vivo repeated dose systemic toxicity testing’ with a total budget of EUR 50 million. Cosmetics Europe published its financial commitment to the Research Initiative at the same time. EUR 25 million funding are provided by the FP7 HEALTH theme and EUR 25 million by Cosmetics Europe.

Recent Developments

During the second Annual Meeting of the SEURAT-1 Research Initiative in Lisbon, Cosmetics Europe published a press release announcing a EUR 8 million extension of its research programme to fund alternatives in animal testing. The following statements were directly taken from this press release, the full text is available online:


The new funds come on top of the EUR 50 million programme that the European Commission and Cosmetics Europe have jointly invested in SEURAT-1 under the 7th Framework Programme, and is also over and above the EUR 12 million committed by the European cosmetic industry in 1997.

The Cosmetics Europe Alternatives to Animal Testing (AAT) extended programme will focus on:
Pre-validation of 'promising' toolbox test methods for Skin Sensitization and data integration activities

Finalising development and conduct pre-validation of the already developed 3D-model for genotoxicity, and promote regulatory acceptance in this field

Refinement of eye irritation assays to address last remaining gaps

At the opening of the conference, Cosmetics Europe Director-General, Bertil Heerink, said: “We need to extend this research programme to take full benefit of all the efforts made in previous years. We want the Cosmetics Europe programme to maintain momentum. This industry continues to be totally committed to replacing alternative methods as soon as scientifically possible and therefore we continue to invest in research. The acknowledgement of our industry’s efforts helps us to continue our research on such a large scale.” (Cosmetics Europe statement, 8 February 2012; www.cosmeticseurope.eu).

These activities are obviously not directly related to repeated dose systemic toxicity, but it is important to note that other fields of research could successfully progress in the development of innovative testing strategies, and that these efforts find acceptance by the regulatory bodies, allowing transition into pre-validation state. However, the field of repeated dose systemic toxicity still requires fundamental research to reach this stadium, and the SEURAT-1 Research Initiative is an important cornerstone in this context, but at the same time it is evident that further efforts complementing the SEURAT-1 activities are needed.

Further funding for such complementary projects are currently being prepared, as outlined in an orientation paper written in connection with the 2013 Work Programme in the area of Health research. Following the recommendation of the EPAA workshop “Harnessing the Chemistry of Life: Revolutionising Toxicology”, a new call for proposals entitled “Modelling toxic responses in case studies for predictive human safety assessment” may be published soon under the HEALTH Theme of the 7th European RTD Framework Programme. The main objective of this topic will be a case study exploitation of recent advances in computational chemistry and systems biology in order to provide the basis for innovative approaches to predictive human safety assessments. However, the working document is not yet endorsed by the European Commission and the final adoption and the publication of the respective work programme are expected in mid-July 2012 via the usual participant portal.

All these new developments underline the importance for starting additional joint efforts to accelerate a paradigm shift in toxicology. Indeed, this paradigm shift from empirical in vivo studies to mechanism-based innovative in vitro approaches is on the way, but will require joint efforts of a broad scientific community with complementary knowledge including the fields of systems medicine, personalised medicine, preventive medicine, and others.

2 - https://ec.europa.eu/research/participants/portal/page/fp7_documentation
3 - http://ec.europa.eu/research/participants/portal/page/cooperation
Uniqueness of the SEURAT-1 Research Initiative

The SEURAT-1 Research Initiative is unique in several aspects:

- Joint funding by the European Commission and a specific industrial sector (cosmetics industry / Cosmetics Europe)
- Coordinated cluster of RTD projects
- Support through a data management and servicing project

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

Goals and Objectives

The goal of the 5-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 better understanding of repeated dose toxicity based on in vitro tests and identifying gaps in knowledge, that are to be bridged by future research work. The end result would be testing methods which, within the framework of safety assessment, have a higher predictive value, are faster and cheaper than those currently used, and significantly reduce the use of animal tests.

In the first Annual Report, the objectives were formulated closely to the expected outcomes of the projects. In the meantime, the projects have not only started their research work, but also established collaborations on various levels and, as a consequence, agreed about the definition of cluster level objectives, which cannot be achieved by the individual projects. These cluster level objectives are:

- to develop highly innovative tools and methodology that can ultimately support regulatory safety assessment
- to formulate and implement a research strategy based on generating and applying knowledge of mode-of-action
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 includes the development of organ-simulating devices, the use of human-based target cells, the identification of relevant endpoints and intermediate markers, the application of approaches from systems biology, computational modelling and estimation techniques, and integrated data analysis.

Structure of the SEURAT-1 Research Initiative

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

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

- ‘Stem Cells for Relevant efficient extended and normalized TOXicology’ (SCR&Tox)
  Scientific coordinator: Marc Peschanski, INSERM/UEVE 861, I-STEM/AFM, Evry / France

- ‘Hepatic Microfluidic Bioreactor’ (HeMiBio)
  Scientific coordinator: Catherine Verfaillie, Interdepartmental Stem Cell Institute, Katholieke Universiteit Leuven / Belgium

- ‘Detection of endpoints and biomarkers for repeated dose toxicity using in vitro systems’ (DETECTIVE)
  Scientific coordinator: Jürgen Hescheler, Institute for Neurophysiology, University Hospital Cologne / Germany

- ‘Integrated In Silico Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety’ (COSMOS)
  Scientific coordinator: Mark Cronin, School of Pharmacy and Chemistry, Liverpool John Moores University / United Kingdom

- ‘Predicting long term toxic effects using computer models based on systems characterization of organotypic cultures’ (NOTOX)
  Scientific coordinator: Elmar Heinzle, Biochemical Engineering, Saarland University, Saarbrücken / Germany
‘Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology’ (ToxBank)
Scientific coordinator: Barry Hardy, Douglas Connect, Zeiningen / Switzerland

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

‘Coordination of projects on new approaches to replace current repeated dose systemic toxicity testing of cosmetics and chemicals’ (COACH)
Coordinator: Bruno Cucinelli, ARTTIC, Paris / France.

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

An example for the scientific management and coordination is the development of an approach for a strategic review at the cluster level: Key contributions of the research projects, which are essential to meet the above-mentioned cluster level objectives, were identified as the starting point for the strategic review (see Figure 1 in the Executive Summary). They will be used to define the cross cluster milestones and to identify connections between the different projects and, thus, form the basis for dynamic networking activities within the SEURAT-1 Research Initiative. The overall approach for this strategic review is currently being developed by the coordination action project COACH in close cooperation with the project coordinators, and the endorsement of this approach by the SEP is expected in the following meetings.

The Annual Report: Something about ‘Pathways’

This is the second 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. This second volume will focus on highlights from the first work period in the research projects and first steps towards reaching the final goal of the cluster. All six volumes together will provide a complete overview about recent cutting-edge research ‘towards the replacement of in vivo repeated dose systemic toxicity testing’ and, thus, represent a ‘pathway’ regarding scientific progress.

This leads to the common theme running through the Annual Report as well as through the SEURAT-1 Research Initiative, as introduced in the first volume: The structure of the Annual Report, which will be kept over the six-year period, is inspired by one of the most important keywords of the addressed field of research, which is “toxicity pathways” (Figure 1.1).
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 second volume it will further elaborate on the hypothesis-driven approach to elucidate mode-of-action and identify key events, effects and biomarkers. This chapter is followed by the detailed project descriptions in chapter 4 that provides an overview about research highlights in the past year. Finally, chapter 5 will focus on the related International Activities and identify potential interfaces in order to establish collaborations for future research and development work leading to pathway based human safety assessments in the field of repeated dose systemic toxicity testing of chemicals.

Overall, conceptual considerations related to biological pathways leading to toxicity will consistently guide through 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 of processes in repeated dose toxicity.

**Network of signalling molecules modulating a “Toxicity Pathway”**

**Book Structure**

*Figure 1.1* The concept of “Toxicity Pathways” is mirrored by the book structure (right panel). In this second Annual Report we show the second layer of complexity through cross-talk between various signalling molecules (nuclear receptors) and a central “Toxicity Pathway (Wnt-pathway, left panel).

**The Consortium and the Scientific Expert Panel (SEP)**

The SEURAT-1 Research Initiative combines the research efforts of over 70 European
universities, public research institutes and companies. The composition is unique, as toxicologists, biologists from different disciplines, pharmacists, chemists, bioinformaticians and leading experts from other domains work closely together on common scientific objectives. The participation of SMEs in SEURAT-1 is high with 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 coordinators of the six cluster research projects and external experts. The SEP composition was recently changed: one of the SEP members resigned and two new members were invited to join the SEP. The new membership is listed in Table 1.1 (the co-chairs are indicated in bold).

**Table 1.1 Members of the SEURAT-1 Scientific Expert Panel.**

<table>
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<tr>
<th>Participant</th>
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<th>Project</th>
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<td><strong>Project Coordinators</strong></td>
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</tr>
<tr>
<td>Marc Peschanski</td>
<td>INSERM/UEVE 861, I-STEM/AFM, Evry /France</td>
<td>SCR&amp;Tox</td>
</tr>
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<td>Mark Cronin</td>
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<tr>
<td>Catherine Verfaillie</td>
<td>Interdepartmental Stem Cell Institute, Katholieke Universiteit Leuven / Belgium</td>
<td>HeMiBio</td>
</tr>
<tr>
<td>Jürgen Hescheler</td>
<td>Institute for Neurophysiology, University Hospital Cologne / Germany</td>
<td>DETECTIVE</td>
</tr>
<tr>
<td>Elmar Heinzle</td>
<td>Biochemical Engineering, Saarland University, Saarbrücken / Germany</td>
<td>NOTOX</td>
</tr>
<tr>
<td>Barry Hardy</td>
<td>Douglas Connect, Zeiningen / Switzerland</td>
<td>ToxBank</td>
</tr>
<tr>
<td><strong>External Experts</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gabrielle Hawksworth</td>
<td>Division of Applied Medicine, University of Aberdeen / UK</td>
<td></td>
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<tr>
<td>Ian Cotgreave</td>
<td>AstraZeneca Safety Assessment, Södertälje / Sweden</td>
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<tr>
<td>Roger Arnold Pedersen</td>
<td>Laboratory for Regenerative Medicine and Cambridge Stem Cell Initiative, University of Cambridge / UK</td>
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<tr>
<td>Hans Juergen Ahr</td>
<td>Bayer Health Care AG, Wuppertal / Germany</td>
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<tr>
<td>Catherine Mahony</td>
<td>Cosmetics Europe; (Procter &amp; Gamble), London Innovation Centre / UK</td>
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<tr>
<td>Derek Knight</td>
<td>European Chemicals Agency, Helsinki / Finland</td>
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<tr>
<td>George Daston</td>
<td>Procter &amp; Gamble, Product Safety and Regulatory Affairs, Cincinnati / USA</td>
<td></td>
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<tr>
<td>Russel Thomas</td>
<td>The Hamner Institute for Health Sciences, Research Triangle Park / USA</td>
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Validation of alternative methods and the subsequent acceptance by regulators are a complex and slow process, e.g. in ongoing ingredient reviews ... Integrated Testing Strategies are a possibility to make most efficient use of alternative approaches.”

2.1 Introduction

The 7th Amendment to the Cosmetics Directive introduced a number of key requirements related to animal testing, which have been incorporated into the Cosmetics Regulation (Regulation (EC) No 1223/2009, 30 November 2009). 2004 marks the introduction of a ban on animal testing of cosmetic products within the EU. In 2009 an EU testing ban for cosmetic ingredients came into force with an extension until 11 March 2013 for three specific areas (repeated dose toxicity (includes skin sensitisation, carcinogenicity and sub-acute/sub-chronic toxicity), reproductive toxicity (also includes teratogenicity) and toxicokinetics).

In the first volume of the SEURAT-1 Annual Report, the status of repeated dose toxicity testing in the context of safety assessment for cosmetic ingredients was described. This chapter is intended to outline the recent developments in legislation regarding chemicals, with a special emphasis on cosmetic ingredients. Secondly, it illustrates the regulators’ needs for acceptance of non-standard methods and, finally, provides an overview about the development of new scientific tools for risk assessment in the field of repeated dose systemic toxicity, and how these developments are eventually related to the SEURAT-1 Research Initiative.

2.2 European Legislation as a Driver for Alternative Approaches to Animal Testing Research

Rob Taalman

2.2.1 Horizontal Legislation

The protection of animals used for scientific purposes: Directive 2010/63/EU revising Directive 86/609/EEC on the protection of animals used for scientific purposes was adopted on 22 September 2010. The Directive is firmly based on the principle of the ‘Three Rs’, to replace, reduce and refine the use of animals used for scientific purposes. The scope is now wider and includes foetuses of mammalian species in their last trimester of development and cephalopods, as well as animals used for the purposes of basic research, higher education and training. It lays down minimum standards for housing and care, regulates the use of animals through a systematic project evaluation requiring inter alia assessment of pain, suffering distress and lasting harm caused to the animals. It requires regular risk-based inspections and improves transparency through measures such as publication of non-technical project summaries and retrospective assessment. The development, validation and implementation of alternative methods is promoted through measures such as establishment of a Union reference laboratory.
for the validation of alternative methods supported by laboratories within Member States and requiring Member States to promote alternative methods at national level.

2.2.2 Sectoral Legislation

Testing needs for the REACH legislation: As an enormous investment into consumer product safety, the REACH programme aims to assess existing chemicals that have previously undergone very little testing. Regulation (EC) 1907/2006, known as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals), revises the Dangerous Substances Directive (67/548/EEC). The registration process has only recently begun, and the estimated testing demands are under debate. However, there is little doubt that systemic toxicity will account for a considerable proportion of the testing costs and animal use of REACH because registration for high and medium tonnage substances must include proposals to fill data gaps for higher-tier toxicological studies. However, ECHA (the European Chemicals Agency) emphasizes that conducting new studies, particularly animal studies, should be a last resort. Instead, registrants should consider using “non-standard data” including existing data, weight of evidence, QSAR, \textit{in vitro} methods, and chemical groupings and read-across approaches, which may provide adequate information and hence be acceptable. While registrants are encouraged to consider all of these options, they must provide robust scientific arguments to justify the use of non-standard data. This is discussed more fully in chapter 2.3, ‘Use of Non-Standard Methods in Regulatory Science: Challenges and Opportunities Illustrated by REACH’ and may to some extent stimulate the development of alternative approaches, especially for systemic toxicities.

Cosmetics regulation – 7th amendment: On January 15, 2003, the EU passed a law banning the testing of cosmetics and their ingredients on animals, reinforced by marketing bans with different deadlines. Known as the 7th Amendment (Directive 2003/15/EC) to the Cosmetics Directive (Directive 76/768/EEC), this Directive is intended to protect and improve the welfare of animals used for experimental purposes by promoting the development and use of scientifically valid methods of alternative testing. The main objective of this Directive is to prohibit the testing of cosmetic products/ingredients on animals through a phased series of EU testing and marketing bans. This ban on animal testing and sales would start immediately where alternative non-animal tests are available, followed by a complete testing ban six years after the Directive became effective (i.e., in 2009). Therefore, animal experiments for cosmetic products and ingredients are completely banned, reinforced with a marketing ban in the EU since 2009, irrespective of the availability of animal-free methods, except for repeat-dose toxicological endpoints (i.e., toxicokinetics, repeated dose toxicity, skin sensitization, carcinogenicity, and reproductive toxicity) where the EU marketing ban is delayed until 2013 for tests carried out outside the EU.
However, the regulation called for a review of the technical feasibility 2 years prior to the implementation of the full ban. This review included an expert judgement on the state of the science on the availability of non animal approaches for the relevant toxicological endpoints and is discussed more fully further in this chapter – the report concluded that in the areas of repeated dose toxicity, carcinogenicity, reproductive toxicity and toxicokinetics, no timeline for full replacement of animals could be foreseen. The only human health endpoint for which a full replacement between 2017 and 2019 may be scientifically feasible is skin sensitisation (allergy), where several non-animal test methods are under development (Adler et al., 2011).

Another study by the European Commission on the possible socio-economic impact of the implementation of the ban is expected to be finalised mid 2012 – this report will cover a number of legislative options (including the full implementation of the ban) to illustrate the type and scope of impact. The outcome of this exercise will help the EC to prepare a proposal for consideration by the European Parliament and the Council of ministers.

The proposal is likely to contain elements that provide an incentive to continue to invest in research on alternatives and to stimulate innovation in Europe.

**Non animal testing provisions in non – EU regulatory schemes:** In many countries formal toxicology trials involving live animals for the purpose of safety testing of cosmetic products and ingredients are required by law. However pressure is building in a number of regions to allow non-animal testing alternatives for cosmetics. The driver for this change is different per country; both economic and ethical considerations play a role. Some examples of countries where non-animal testing is under debate are listed below.

A recent example of such change comes from China. In February 2012, China’s State Food and Drug Administration, or SFDA, released a draft proposal approving the use of alternatives to animal testing for cosmetics. This is the first time the SFDA, which oversees the testing and registration of all domestically and internationally produced cosmetics on sale in China, has seriously considered changing its regulations to allow non-animal testing alternatives for cosmetics. The method being considered for adoption is the 3T3 NRU phototoxicity test method which is already widely accepted internationally.

Also in the USA, animal testing has recently become a hot topic, where a class action lawsuit was filed in the central district of California on Feb. 28, 2012. This might renew the discussion in the USA on acceptance of alternative methods for safety assessment of cosmetics.

Last year (September 2011) an agreement was signed in Brazil by Anvisa (National Sanitary Surveillance Agency) with the goal to carry out less pre-clinic or safety tests on animals. The emphasis is on safety testing for the registration of vaccines, pharmaceuticals, cosmetics, food and cleaning products. The cooperation agreement was signed with a unit already conducting research in the area.
2.2.3 Conclusion

Non-animal testing provisions continue to provide an incentive to continue to invest in research on alternatives and to stimulate innovation in Europe and pressure is building in a number of regions outside of the EU to allow non-animal testing alternatives for cosmetics. The driver for this change is different per country; both economic and ethical considerations play a role.

2.3 Use of Non-Standard Methods in Regulatory Science: Challenges and Opportunities Illustrated by REACH

Derek J Knight

2.3.1 Information on Substance Properties Illustrated by REACH

Toxicological, ecotoxicological, environmental fate and physico-chemical properties of chemicals have to be determined for safety assessment and regulatory approval. Traditionally such hazardous properties are determined by testing using standard laboratory studies conducted in compliance with Good Laboratory Practice (GLP). These studies are in effect used to model the impact that the chemical substance has on human health.

The various regulatory schemes for chemical and other specific products have their own data requirements, including mechanisms for varying the standard testing. One illustration is the EU scheme to control chemicals, i.e. the Registration, Evaluation and Authorisation of Chemicals (REACH) Regulation (Anonymous, 2006). Chemical substances manufactured or imported, either neat or in a preparation, at > 1 tonne per annum have to be registered. The information on hazardous properties is linked to the manufacture or import level, on the grounds that there is a potential for more exposure as more substance is manufactured or imported in the EU.

2.3.2 Non-standard Data for Use within the Framework of REACH

The registrant can ‘adapt’ the standard information requirements under REACH, and use other information instead: non-standard or non-GLP studies, in vitro studies, human epidemiology data, information from structurally-related substances (i.e. ‘read-across’ and ‘chemical categories’), predictions from valid (Q)SARs and use of the weight of evidence (WoE) approach. The non-standard information has to be equivalent to the information obtained from the standard studies, in that the key parameters of the standard method should be addressed
and the result must be suitable for adequate risk assessment and/or classification under the Classification, Labelling and Packaging Regulation (CLP; Anonymous, 2008). Registrants have to justify these adaptations of the standard information requirements in the registration dossier by providing scientific explanations; for example there should be a hypothesis which justifies why the properties of a substance can be ‘read across’ to another substance.

There are various issues for debate in regulatory science on how to use non-standard methods to assess the properties of substances, e.g.:

- Should predictions of ‘simple’ properties be regarded differently to ‘complex’ endpoints; i.e. are some endpoints intrinsically more ‘difficult’ to predict than others?
- Should ‘borderline’ predictions close to a regulatory threshold be regarded differently?
- Are predictions of ‘negative’ effects regarded differently to those predicting ‘positive’ effects?
- Should predictions based on a mode (or mechanism) of action be regarded as better than those based on correlation alone?

2.3.3 Data Waiving Approaches and REACH

The standard set of toxicological studies for various regulatory schemes can be very extensive and use many experimental animals. Hence before conducting new studies it is important to consider potential ‘data waivers’. These are regulatory justifications to omit particular studies on the grounds that:

- It is impossible to conduct the study for technical reasons.
- It is scientifically unnecessary as the result can be predicted by other means (as discussed above).
- The study is not necessary for risk assessment on the grounds of ‘low’ exposure.

This latter consideration of low exposure is illustrated by two provisions in REACH:

- ‘Substance-tailored exposure-driven testing’ to allow for reduced toxicological testing, if justified by the registrant, of substances manufactured and used under conditions resulting in no significant exposure.
- Registration of chemical intermediates that are manufactured and used only under ‘strictly controlled conditions’ with only existing studies (or physicochemical properties tests in some circumstances).
The same concept of judging that risk is adequately controlled without (full) testing is applied in other regulatory fields using the regulatory approach referred to as the Threshold of Regulatory (or Toxicological) Concern.

2.3.4 **Intelligent Approach to Property Evaluation**

New animal studies should only be conducted for REACH registration as a last resort and there are data sharing obligations for registrants of the same substance to avoid duplicate testing. Registrants must first collect and assess all existing data, then identify data gaps and consider whether they can be filled by non-standard data before deciding on new studies. This is, in effect, an intelligent and tiered approach to testing based on these principles:

- Making maximum use of existing studies and literature data.
- Predicting properties using calculation, estimation or ‘analogy’ to tested substances.
- Using data waivers to justify omitting studies for technically-impossible studies, scientifically-unnecessary studies or on the grounds of low exposure.
- Conducting appropriate non-animal tests.
- Adopting a WoE approach.

2.3.5 **Risk Assessment Using Non-standard Data**

Risk assessment is central to the operation of REACH: industry conducts a Chemical Safety Assessment, in the form of a Chemical Safety Report (CSR) for substances at above 10 tonnes per annum. ‘Exposure scenarios’ (ESs) are the key outputs, as a description of manufactured/use in the form of ‘operational conditions’ (OCs) linked to ‘risk management measures’ (RMMs).

For the human health risk assessment a key concept is the ‘derived no effect level’ (DNELs), i.e. the level below which adverse effects are not anticipated for a particular exposure route and duration. DNELs are estimated for the various exposed human populations, based on the appropriate toxicity data set and using ‘assessment factors’. Thus conceptually, animal toxicology studies are used to model effects in humans by applying assessment factors to take account of the associated uncertainties. The risk assessor can deviate from the standard assessment factors on a case-by-case basis to take account of extra uncertainty from the data set used, e.g. because non-standard information such as ‘read across’ data are to be used, but such adjustments must be justified and recorded in the CSR. The risk assessor should apply good science and use professional judgement in assessing the hazardous properties of substances and assessing the risks from their manufacture and use.
2.3.6 Speculations on Developments in Regulatory Science on Property Assessment Techniques

Finally it is interesting to speculate on possible future developments in regulatory science that could be of practical use in the medium term for improving and further developing the current techniques for regulatory hazard and risk assessment of chemicals. It would be good to ‘add value’ by building on and integrating current methodologies to produce scientifically-valid and generally-accepted and ‘fit for purpose’ techniques that are fairly standardised and documented (yet still flexible) for use by industry and regulators in assessing the properties of substances and undertaking risk assessments. To support devising such combined approaches, further work may need needed into the underpinning biological mechanisms that determine toxicity from chemicals. Measures to assess the uncertainty in the predicted properties will be important, with the idea that risk assessment methods could be deployed to deal with this uncertainty yet still deliver an outcome that is suitable for regulatory decision making. Some suggestions are to:

- Develop practical approaches incorporating combinations of existing methods and tools to devise approaches that have international scientific and regulatory acceptance, e.g. as ‘test batteries’, ‘toolkits’ and ‘integrated strategies’.

- Look for opportunities to use non-test data in specific circumstances, e.g. use higher assessment factors in risk assessment to take account of higher uncertainty in property prediction.

- Make better use of existing data, both to support read-across/categories and also to develop other prediction methodologies, such as improving QSARs and expanding the chemical domains which apply.

- Make use of information from ‘new approach’ toxicology assessment paradigms and ‘high throughput screening’ approaches (e.g. EU SEURAT-1 & US EPA ToxCast), perhaps to support and strengthen the hypothesis for read-across and chemical categories in a WoE approach.
References


2.4 State of the Science in Innovative \textit{in vitro} and \textit{in silico} Methods for Repeated Dose Systemic Toxicity Testing

Catherine Mahony

2.4.1 Perspectives for the Replacement of Animal Testing in the Field of Repeated Dose Toxicity Testing

In 2010, a panel of scientific experts commissioned by the European Commission described the current status and future prospects for alternative (non-animal) methods for cosmetic testing (Adler et al., 2011). In essence, they were unable to estimate the time horizon for full replacement of repeated dose toxicity testing (and carcinogenicity and reproductive toxicity too) because the research is not sufficiently advanced and complex mechanisms and biological systems which are the basis for systemic toxicity are only poorly defined at present. This knowledge of biological systems, mechanisms of toxicity, and interactions among systems is necessary to make sense of non-animal data as predictors of risk for human toxicities. Equally important is an understanding of the concentrations at which adverse effects happen and of ‘safe’ doses, which relies on the ability to extrapolate from \textit{in vitro} to \textit{in vivo} to determine how this relates to consumer exposure. For this area of toxicokinetics a time frame was estimated of 5-7 years to improve existing models and develop models to predict lung absorption and renal/biliary excretion which are priority areas for cosmetic ingredients considering the significance of the dermal route of exposure for a large number of cosmetic products. It was proposed to take even longer to integrate the methods to fully replace animal testing, whereby the results from \textit{in vitro}/\textit{in silico} testing are linked with toxicokinetic modeling. No specific time frame could be given by the experts here. Also important to note is that the timeframe for any sort of validation (pre or otherwise) and regulatory acceptance of methods was not included although a further 4-8 years was put forward to account for this, assuming resources are available and a successful outcome is reached.

Following publication of this report, an independent evaluation was undertaken in 2011, by a transatlantic think tank for toxicology-t4 (Hartung et al., 2011). The reviewers agreed with the overall conclusions of the Adler report and endorsed the current lack of availability of a full replacement especially in the area of repeated dose toxicity, carcinogenicity and reproductive toxicity. Of particular note for these reviewers, already one year on was an emerging roadmap, but an emphasis on strategic planning and sustained efforts was highlighted as necessary, particularly to take account of more global collaborations and the inclusion of other industrial sectors. Others too have offered such opinion, highlighting the need to better enable ‘coordination’, greater outreach and communication across stakeholder communities (Martin et al., 2012).
2.4.2  

\textbf{In silico Methods: Physiologically-based Pharmacokinetic Models and Structure-Activity Relationships}

The Hartung report was able to build further on discussion of the importance of bioavailability prediction models, namely to take account of various factors that may quantitatively affect exposure as well as the ability to model a chemical dynamically as well as kinetically. In the same way that the safety assessor today has to characterise exposure and all factors that may impact on it, this thinking will be needed also for application of in vitro test data to 21st century risk assessment. The visibility of PBPK-modeling to provide points of departure for establishing safe exposure levels (in vitro to in vivo dose-response curves) is indeed growing (Ans et al., 2011). Efforts by ToxCast in collaboration with the Hamner Institute to relate chemical exposure to blood concentrations are of interest here. Experimental measurements of plasma protein binding and ability of human liver cells to metabolise a given chemical were made for 239 chemicals. This enabled conversion of in vitro concentrations into oral equivalent doses, i.e. doses necessary to produce steady-state in vivo blood concentrations equivalent to in vitro AC(50) (concentration at 50% of maximum activity) or lowest effective concentration values across more than 500 in vitro assays. These oral equivalent doses were then compared with chronic aggregate human oral exposure estimates to assess whether in vitro bioactivity would be expected at the dose-equivalent level of human exposure and to aid prioritisation of chemical testing programmes (Wetmore et al., 2011).

Application of PBPK modelling to in vitro toxicity testing and human risk assessment is also highlighted in a couple of choice papers respectively; i) Simulation of concentrations for dosing in an in vitro study was shown to be representative of concentration-time profiles following oral dosing of a defined set of compounds (n=29), taking account the percentage absorbed, and considered by the authors to be better than using data on cytotoxicity from in vitro studies (Hans et al., 2011). Kinetic profiles differed according to blood: tissue partitioning and maximal concentrations depended mainly on the dose and fraction absorbed ii) An approach to predict in vivo dose response curves for human developmental toxicity of glycol ethers was demonstrated by Jochem et al. (2010) by combining in vitro toxicity data and in silico kinetic modelling.

Further methods highlighted in the Hartung report such as TTCs as well the importance of being able to harness available data are notably both a key focus of the SEURAT-1 Research Initiative vis-à-vis COSMOS and ToxBank. Others have also recommended a reliable open curated database that interfaces with existing databases to enable sharing of information (Silbergeld et al., 2011), notably a primary goal of ToxBank, within the SEURAT-1 Research Initiative. Liver cell co-cultures, expanded cell types from stem cells and more ‘-omics’-based technologies were also amongst additional suggested methods in the Hartung review, and are the focus of SCR&Tox, HeMiBio, DETECTIVE and NOTOX within the SEURAT-1 Research Initiative. SEURAT-1 is however recognized as just a first phase and expansion will surely be
needed. Whilst key elements of ‘what next’ has already been highlighted in the first Annual Book it will be critically important to stay abreast of scientific advances in the field of systemic toxicity alternatives, both at a cluster level and undoubtedly as highlighted in the t^6 report to take account of methods and research activities at an international level.

One such emerging field is in the area of Structure Activity Relationships (SAR). These are qualitative assessments, involving expert judgment of structural features, assessment of factors that affect ADME and consideration of other supportive information (chemical and biological activity) as a basis for prediction of toxicity. QSAR on the other hand is a quantitative method which involves development of mathematical models that relate the biological activity of molecules to their chemical structures and corresponding physicochemical properties and other molecular properties. In silico tools have their limitations and strengths and an understanding of the different parameters is necessary to make best use of these tools. Modi et al. (2012) have recently proposed an integrated workflow for combined use of data extraction, QSARs and read across methods, and have highlighted how this can enable transition to Toxicity Testing in the 21st Century. In the case of SAR, Wu et al. (2010) have proposed a framework for evaluating the suitability of analogs for SAR assessment. The analogs are then ranked on the basis of their suitability and undergo a chemistry evaluation, metabolism evaluation and toxicity data review and uncertainty ranking. Uncertainty ranking is impacted by number of analogs and their suitability ratings, biological concordance across analogs, corroborating metabolism data, corroborating data on structure of interest for one or more endpoints, mode of action for analogs, quality of the study data. Ultimately this allows for expression of the confidence in the degree of uncertainty as shown below in Table 2.1.

**Table 2.1** Toxicity data review and uncertainty ranking as it relates to SAR assessment

<table>
<thead>
<tr>
<th>High Uncertainty</th>
<th>Moderate Uncertainty</th>
<th>Low Uncertainty</th>
</tr>
</thead>
<tbody>
<tr>
<td>Read Across Not Recommended</td>
<td>Read Across may be possible for some endpoints-larger MoE^1 required</td>
<td>Read Across does not require a larger MoE</td>
</tr>
</tbody>
</table>

^1 Margin of Exposure

14 blinded case studies were then progressed by expert reviewers across a series of endpoints which determined read across results supported by the ‘process’ for these blinded case study chemicals to be protective when compared to the bon fide toxicity data on the case study chemicals. I.e. Genetic Toxicity +/- (all correct predictions), Repeated Dose Toxicity (surrogate NOAEL estimate) (no under estimates), Developmental Toxicity (critical effect +/-, if yes surrogate NOAEL) (no under estimates) and Reproductive Toxicity (critical effect +/-, if yes surrogate NOAEL) (no under estimates) (Blackburn et al., 2011).

The authors concluded that this process for safety assessment based on read across can be
successfully applied to develop surrogate values for risk assessment. They cautioned however
that successful application of the approach requires significant expertise as well as discipline
to not over step the boundaries of the defined analogs and the rating system. The end result
of this rigor may be the inability to read across all endpoints for all chemicals resulting in data
gaps that cannot be filled using read across. One can envisage that additional confidence in
SAR assessment would be gained via additional work to model PBPK (parent compound and
actives metabolites) combined with \textit{in vitro} toxicity test data and if truly necessary that such a
data package would support limited and targeted \textit{in vivo} toxicity testing.

Future work is mentioned by Wu et al. (2010) that will focus on additional flags for
developmental/reproductive toxicity (DART). Such an exercise offers promise in terms of its
utility to define rules that associate molecular features with toxicity and will in turn begin to
reveal a compendium of known mechanisms of toxicity. Not only will this serve to inform
toxicity mechanisms beyond those based on reactive chemistry, it also offers opportunity for
expansion and exploitation in efforts to describe ‘adverse outcome pathways’. A necessary step
towards fulfilling ‘mapping of the human toxome’ as coined by Hartung & McBride (2011). In a
project of the International QSAR Foundation it is envisaged that adverse outcome pathways
will be described in an encyclopedic manner in Effectopedia, an open knowledge aggregation
and collaboration tool\footnote{1 - http://blog.okfn.org/tag/effectopedia/}. It is also foreseen that adverse outcome pathways will serve a role in
helping to formulate mechanistically relevant chemical categories, by integrating knowledge
of how chemicals interact with biological systems (i.e., the molecular initiating events) and \textit{in vitro}
and \textit{in vivo} knowledge of the biological responses\footnote{2 - http://alttox.org/spotlight/050.html}. Clearly elucidation of mechanisms
of toxicity and how adverse outcome pathways interplay with each other will be an iterative
process of discovery, learning and application.

### 2.4.3 Experimental Approaches for the Elucidation of Toxicity Pathways

There is common agreement amongst the scientific community that mechanism centred toxicity
testing is currently hampered by our current understanding of toxicity pathways and yet such
understanding is a necessary prerequisite to the development of assay systems for repeated
dose toxicity testing. The use of toxicogenomics has been highlighted as an important data
stream to aid identification of mechanisms of actions and to make predictions on toxicity as
demonstrated by the work of (Daston & Naciff (2010) in the area of developmental toxicity
and by Fielden et al. (2011) in the area of nongenotoxic hepatocarcinogens. The expanded
use of data streams such as toxicogenomics to identify the essential cellular components and
pathways involved in the toxicity response and combination with mechanism centred targeted
assays (taking account of metabolic activation) is believed to be the approach needed to meet
the challenge of toxicity testing in the 21\textsuperscript{st} Century (North & Vulpe, 2010; Mahadevan et al.,
2011).
qHTS screening is also a valuable tool, playing a pivotal role in the Tox21 programme in profiling compounds tested in the Tox21 screening assays. To date hundreds of thousands of compounds having been run through qHTS screening (titration based) assays at the NIH Chemical Genomics Center (NCGC) (Shukla et al., 2011). Huang et al. (2011) have furthermore demonstrated the feasibility of qHTS to identify the potential of environmental chemicals to interact with human nuclear receptors, finding better concordance for agonist mode than for antagonist mode (likely attributable to the interference of cytotoxicity in the latter assays). The authors were also able to formulate data driven strategies for discriminating true signals from artefacts and to prioritise assays based on data quality.

The recent emergence of pathway approaches has also highlighted the progression of Toxicity Testing in the 21st Century. For example an adverse outcome pathway perspective of embryonic vascular development can help identify useful information for assessing adverse outcomes relevant to risk assessment (Knudsen & Kleinstreuer, 2011).

A proposed potential testing scheme for nanomaterials that works towards an integrated testing strategy has focussed also on pathways of toxic responses (Silbergeld et al., 2011). Elucidation of the molecular mechanisms of reference materials for specific nanomaterial classes/subclasses is proposed using short term in vivo animal studies in conjunction with High Throughput screenings and mechanisms based short term in vitro assays. The importance also of physicochemical parameters is stressed as contributing to toxicity of nanomaterials and thus their role to aid in the prediction of hazard potential of certain nanoparticles based on property-activity relationships (Lai, 2012).

Although the emphasis on Toxicity Testing in the 21st Century is on improving risk assessment as it relates to human health endpoints, the above mention of targeted animal testing to aid understanding on the roles of specific genes in biological pathways and systems does not standalone. Transgenic animal models have been highlighted as a powerful tool for developing this understanding, and have been proposed as an integral tool for toxicity testing in the 21st century (Boverhof et al., 2011). Interestingly a cross-species comparison (zebrafish embryos, rats and rabbits) has implied a common basis for biological pathways associated with neuronal defects, extracellular matrix remodelling and mitotic arrest (Sipes et al., 2011). Revealing this sort of perspective further for other pathways and in particular for human health endpoints would be expected to target any in vivo toxicity testing that may be considered necessary, until such time that animal testing can be fully replaced.

The need for ‘proof of concepts’ and ‘case study’ approaches has been commonly highlighted to accelerate toxicity testing in the 21st Century, using well-studied prototype compounds with known toxicity pathway targets (Martin et al., 2012), such as DNA damage and repair (Andersen et al., 2011; Bhattacharya et al., 2011) and the Nrf2 antioxidant pathway (Krewski et al., 2011; Berg et al., 2011). Such approaches are however not unique to Tox21 and have been foreseen to directly inform and complement the SEURAT-1 Research Initiative.
2.4.4 **Towards Integrated Testing Strategies**

The Hartung review lays out the challenging task to specify research milestones for easy comparison of the work ahead and to develop an overall approach (decision tree) to integrated testing strategies.

The strategic review of **SEURAT-1** (see Executive Summary) highlights cross cluster critical key contributions from the cluster projects, which can and should be used for easy analysis of the work underway and for comparison to other initiatives, to identify synergies and foster collaborations. An initial proposal for an overall approach ultimately for the purpose of safety assessment in the absence of animal test data is shown in *Figure 2.1*.

**Figure 2.1 SEURAT in practice for cosmetic ingredients?**

These basic steps in the process of safety evaluation in the absence of animal test data need to be expanded on to (i) further elucidate how it can be done, (ii) formulate the critical research questions and (iii) identify knowledge gaps. In other words, to generate the tools for the tool box, appropriate to the stage of the assessment process. As pointed out by Berg et al. (2011) method harmonization and standardization as well as procedures and guidelines for pulling together ITS is the way forward. When all is said and done though, the new paradigm for toxicity
testing does not appear so very different from the widely used four-stage risk assessment framework originally proposed by the National Research Council’s *Risk Assessment in the Federal Government: Managing the Process*, 1983 *(Krewski et al., 2011)*. Namely hazard identification, dose-response assessment, exposure and risk characterization. We just need to figure out how to make sense of the non-animal test data for hazard characterization and ensure the dose-response from *in vitro* test data is meaningful to the human *in vivo* scenario. The journey along the stony road has begun!

### 2.4.5 Summary

Several overviews of advantages, limitations and development needs for toxicity testing in the 21st Century have been provided but it will remain important to maintain a focus on emerging technologies and the issues that have to be addressed in order to make safety assessment in the absence of animal data a reality. Issues surrounding current animal test methods are likely to apply to the new approaches to toxicity testing, of particular note but not limited to, use of very high dose levels that often far exceed human exposure levels, issues surrounding interpretation of minor effects, difficulties interpreting low incidence findings *(Carney et al., 2012)*.

Central to new approaches to toxicity testing is a mechanistic redefinition of adverse effects based on *in vitro* toxicity testing which will require a series of prototypes to show the process in practice *(Boekelheide & Andersen, 2010)* and although not a current focus of the science, the need for a shift in the current validation paradigm for alternatives to toxicity testing is already apparent with such innovative approaches *(Wilcox & Goldberg, 2011)*.

Developing new strategies for toxicity testing inevitably requires alignment of different areas of science. The SEURAT-1 Research Initiative is well placed in this regard and will be further informed by an upcoming EPAA workshop that with the input of chemists, systems biologists and toxicologists will begin to map out how respective sciences and developments within could be exploited to develop new strategies for toxicity testing, using adverse effects in the liver as an initial focus of attention *(Kimber et al., 2011)*.

Finally we are reminded of an underpinning importance of 21st century science and that is for the benefit of human health, whereby discoveries can be turned into medical benefit *(Cavero, 2011)*.
References


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

“We can't solve problems by using the same kind of thinking we used when we created them.”

Albert Einstein
3.1 Introduction

The guiding principle of the research strategy was outlined in the first Annual Report of the SEURAT-1 Research Initiative. It 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. Hence, this strategy fundamentally relies on harvesting, generating and consolidating knowledge about mechanisms of repeated dose systemic toxicity, and then using this knowledge in a purposeful and rational manner to deliver predictive safety assessment tools. In this second Annual Report we further elaborate on our research strategy and describe how it is being shaped and implemented within the cluster.

3.2 A Mode-of-Action Backbone based on Mechanistic Understanding

The first immediate sign of how the strategy has influenced the cluster is reflected in the approach adopted for the selection of reference chemicals to be used across the projects. A detailed description of the chemical selection procedure is provided in chapter 4.10.2 but is referred to here in the context of the SEURAT-1 research strategy. Chemical selection is never an easy task and is usually exacerbated by a wide variety of criteria and requirements proposed by prospective users. Traditional approaches therefore often set out to select reference chemicals that satisfy a heterogeneous set of criteria such as: belonging to certain chemical classes, used in selected commercial sectors or products, possessing particular physico-chemical properties, or associated with certain adverse health outcomes. Embracing the SEURAT-1 philosophy, the ‘Gold Compound’ Working Group committed instead to first identify and describe a range of known modes-of-action more commonly cited in repeated dose toxicity studies, and then to pick molecules for which there is ample mechanistic evidence of association with toxicological effects or pathways underpinning those modes-of-action. In a second step, criteria of a more practical nature were applied such as commercial availability, cost, and suitability for testing the chemical within an in vitro system. Not surprisingly, many of the reference chemicals are actually pharmaceuticals since these molecules typically have specific mechanisms or modes-of-action which are extensively described in the literature. It is precisely these mode-of-action related properties that make them reliable candidates...
for nomination as reference compounds, rather than their actual origin or commercial use. Therefore once a test system is established to prospectively evaluate if chemicals of interest are causing toxicity through a specific mode-of-action, those chemicals which are found as positive can be considered as being similar with respect to their membership of the same toxicodynamic category or group. This categorisation or grouping therefore has a mode-of-action basis and is populated initially by the associated reference chemicals. This approach doesn’t preclude the possibility that an actual chemical may be promiscuous in nature, being associated possibly with more than one mode-of-action. In this case it is probable that toxicokinetic aspects will play a role in determining which mode-of-action will likely dominate under particular conditions. Moreover, promiscuity at the level of molecular initiation potentially triggering a range of possible modes-of-action may still be reduced to a reasonable number of categories based on a reduced number of possible downstream effects, such as ‘gross’ phenotypic cellular outcomes.

Selecting reference chemicals using a mode-of-action approach also has implications for how they are actually chosen for research purposes. Essentially, SEURAT-1 investigators should first decide on what mode-of-action is of relevance to their particular study or test system, and then select the associated reference chemicals. Thus mode-of-action thinking is brought to the forefront, with the design, optimisation and evaluation of in vitro test systems being driven by the aim to capture one or more specific modes-of-action with high sensitivity and selectivity. As a consequence, the specifications of the biological model, the exposure protocol, the biomarkers to be measured, and the reference chemicals to be used as positive controls, all depend on the mode-of-action chosen.

As introduced in the first SEURAT-1 Annual Report, the identification of modes-of-action and underlying mechanisms that are specific to repeated dose systemic toxicity is a prerequisite for the development of alternative methods in this area (Schwarz & Mahony, 2011). But the knowledge on modes and mechanisms of action of chronic toxicity is highly fragmented and scattered across many different sources, and in many respects is incomplete. Moreover, the manner in which mode-of-action knowledge is described by investigators is highly inconsistent making it difficult to reconcile and consolidate complementary pieces of information to establish a clear and consistent picture of a toxicological process. Another important obstacle too is that many investigators often fail to provide sufficient evidence to support their postulations and thus the validity of the mode-of-action they describe might be questionable.

In order to get a better understanding of the challenges behind mining mode-of-action information and to contemplate the establishment of a focused knowledge-base to serve the cluster, a workshop entitled ‘Mechanisms underlying repeated dose systemic toxicity’ was held on 14-15 November 2011 at the European Commission Joint Research Centre (JRC) in Ispra, Italy. The workshop was organised by the JRC Institute for Health and Consumer Protection and the University of Tübingen, on behalf of COACH, the coordination and support project
of the SEURAT-1 Research Initiative. It brought together highly experienced toxicologists to share selected case studies that describe modes and mechanisms of repeated dose toxicity linked typically to well known ‘model’ toxicants. Taking a case study approach seemed the most practical way to get started since attempting up front to map out a mode-of-action domain that could encompass the whole universe or ontology of toxicological responses was too daunting. The presentation of the case studies also provided the opportunity to appreciate not only the knowledge content in the talks, but also the manner in which the experts conveyed their understanding of typically complex processes using a variety of illustration techniques (see also the workshop report in chapter 4.11.2).

The workshop proved invaluable in identifying what issues need to be resolved if we are to establish a useful mode-of-action knowledge-base, and set the scene for how sufficiently elaborated mode-of-action descriptions could be developed to serve the cluster. For example, it was agreed that since toxicokinetics dictates the magnitude and pattern of internal dosing at target sites, it has considerable influence on which initial toxicological events might be initiated, and in turn, what particular cascade of causally linked downstream events is most likely to follow. Thus toxicokinetics contributes to the ultimate association of a chemical with a specific mode-of-action, and any transitioning between them. A very simple illustration of this is how a cytotoxic agent administered at a high dose can trigger acute organ failure, whereas the same agent given repeatedly at low doses may ultimately lead to fibrosis in the same organ. Another issue that was discussed was the degree of resolution or granularity one should aim for when setting out to describe a mode-of-action and the underlying mechanisms. The conclusion was that it is not a case of ‘one size fits all’ and thus different descriptions of essentially the same mode-of-action might be required to satisfy different purposes. For example, the level and type of detail that one would need to provide a blueprint for building a model for quantitative prediction of an adverse effect will be very different from that used as a basis for a weight-of-evidence argument that a chemical is, or is not, associated with a particular mode-of-action. Better understanding and explanation of in vivo adaptation and repair processes and their intrinsic role in dictating outcome was considered of paramount importance, particularly in the area of repeated dose systemic toxicity. Finally, something as practical as terminology was also highlighted as a potential obstacle to progress since for the moment it would appear that one man’s ‘mechanism’ is another man’s ‘mode’, is another man’s ‘pathway’.

The workshop delivered a number of practical recommendations to be taken up by the cluster in its second year. Overall there was a strong feeling that establishing a mode-of-action framework as a cross-cutting endeavour should be a priority for SEURAT-1 and, thus, measures should be taken to facilitate this. As a follow-up to the workshop therefore, a dedicated breakout session entitled ‘Mode of action – repeated dose systemic toxicity’ was organised at the SEURAT-1 Annual Meeting held in Lisbon on the 8th of February 2012 (a report is given in chapter 4.9.2). Shortly thereafter, reflecting the enthusiasm and commitment shown by many project partners, a specific ‘Mode of Action’ Working Group was established.
– more details about the operation and objectives of this Working Group can be found in chapter 4.10.4. Another recommendation from the November 2011 workshop was that we need to learn by doing, with the starting point being to identify some ‘prototype’ modes-of-action which could be elaborated by dedicated working groups. In this first exercise, designing the processes and techniques to capture and communicate mode-of-action knowledge should be given as much attention as trying to achieve an actual result. This will be taken up in the Mode-of-Action Working Group which has already begun identifying candidate modes-of-action for this first exercise. Of course much can be drawn from the knowledge already gathered by the Gold Compound Working Group and the experience gained in the selection of the reference chemicals.

As yet there is no generally accepted practice for gathering mode-of-action knowledge and presenting it in a consistent and structured manner so that it can be effectively managed and transferred. However, the International Programme on Chemical Safety (IPCS) of the World Health Organisation (WHO) has published guidance (Boobis et al., 2008) on what type of information should be provided to describe a mode-of-action and just as importantly, how should the relevant evidence be presented to demonstrate the validity of the description proposed. More recently, the OECD has followed this direction but gone somewhat further by proposing that mechanistic or mode-of-action information on a chemical can be captured using an analytical tool termed, ‘Adverse Outcome Pathway’, or ‘AOP’. Guidance has been issued on how to practically describe an AOP following a recommended template and also on how to present and evaluate scientific evidence to assess its completeness (OECD, 2012). A supporting document summarising published definitions of relevant terms is also provided to facilitate more transparent communication between different scientific communities and as a step towards eventual harmonisation of vocabulary and definitions. The OECD has also established an AOP work programme to be led by the Advisory Group on Toxicogenomics and Molecular Screening which will officially commence in 2013. Taking these international developments into consideration therefore, the Scientific Expert Panel of the SEURAT-1 Research Initiative has recommended that the Working Group on Mode-of-Action investigate the usefulness of WHO and OECD guidance to serve the needs of SEURAT-1. Moreover, the Mode-of-Action Working Group should also establish links to the WHO and OECD initiatives to identify opportunities for collaboration and to explore how the valuable material already produced could be utilised for our own purpose.
In the following we discuss the critical aspects which must be considered with regard to this overarching goal.

In the selection of relevant modes-of-action, the relevant knowledge will be taken from human or animal data reported in the literature. Other very important resources include publicly available data-bases that can be mined for that purpose. Of particular interest in this context is the ‘OPEN TG-GATES’ database established by the Toxicogenomics Project in Japan. This not only contains information on liver and kidney toxicity of about 150 chemicals tested in rats at 4 different doses including the control and 8 exposure times up to 15 or 29 days, but also the information on global gene expression changes observed in the target organs of the exposed animals. In addition, this database contains information on global gene expression changes in human and rat hepatocytes incubated *in vitro* with the test chemicals. These data can, for example, be used to identify genes clusters showing essentially the same time response across groups of chemicals where the mode-of-action is assumed to be similar, and then to identify putative transcription factors regulating the expression of the gene cluster, and finally to construct gene regulatory networks relevant for the toxicity of interest.

Another highly interesting source of information is the DrugMatrix database of the US National Toxicology Program containing information on more than 600 compounds studied at multiple doses and time points, having a variety of different tissues as targets. Again, this database contains detailed information on histopathological findings and on exposure-related changes in global gene expression which could be mined, for example, to identify molecular events initiating the cascade of higher level effects that ultimately lead to overt toxicity.

In summary, mining of existing databases will strongly assist the identification of the mode-of-action of a given chemical and the upstream molecular events that trigger it.

The beginning of any toxicological pathway or AOP is defined by a molecular initiating event (MIE). It is very likely that there are common MIEs for many pathways, and a threshold value must be reached to significantly disturb a certain pathway. It is, however, *a priori* not clear whether the MIEs relevant for repeated dose systemic toxicity differ from those relevant for acute toxicity, but it is reasonable to assume that there is at least some mechanistic overlap between both exposure scenarios. Most current knowledge of mechanisms of toxicity stems from the acute exposure scenario and thus it will be important to decide whether the same mechanisms also hold true for repeated dose exposures, or whether different or additional mechanisms come into play under the latter condition. Mechanisms to be considered include: (i) repeated hits on the same molecular target, (ii) overload of defence/repair mechanisms, (iii) progressive change in the epigenome, (iv) effects on the immune system such as proliferation.
of memory cells and progressive activation and transformation of hepatic stellate cells, and finally (v), induction of a sequence of adverse reactions involving different cell types (and organs).

An important factor, too frequently ignored, relates to the toxicokinetics of the chemical in question. This can be very different in an in vitro system when compared to in vivo because of, for example, (i) accumulation of a chemical in a target organ due to slow metabolism, (ii) inhibition of an inactivating enzyme, (iii) lowering of metabolic clearance (damage to liver), or (iv) induction of a bioactivating enzyme. It is obvious therefore that the apparent toxicodynamic behaviour of a given compound will be strongly influenced by its toxicokinetics. As a consequence, a central issue for the SEURAT-1 Research Initiative is how to relate treatment concentrations used in the various in vitro test systems to in vivo serum and target organ concentrations, and vice versa.

Given the composition of the SEURAT-1 Research Initiative it is reasonable to start with modes-of-action that are of relevance to the liver and to try to define related pathways based on the identification of interactions of a chemical with known targets. In addition, the potential of a chemical to bioaccumulate at certain initiation sites should be considered as one mechanism producing repeated-dose toxicity. A possible candidate to start with would be a pathway triggered by an alkylating agent such as carbon tetrachloride, as the mechanism of liver toxicity of this class of compound is fairly well understood (carbon tetrachloride itself can probably not be used in vitro because of its high volatility). The pathway description will work in both directions, i.e., even though it is of interest to understand which effects are caused by the alkylans on the organ level, it is mandatory to walk backwards from the adverse effects observed and try to understand which key events lead to this effect, and all the way back to the molecular initiating event. Conceptually, the molecule as such is not of interest, but the pathway it disturbs.

Based on the differences in chemical reactivity it could be useful to describe pathways starting with an irreversible interaction (i.e. caused by highly reactive compounds) and compare them with others starting with a reversible, receptor-mediated interaction. In principle, the two mechanisms can be distinguished experimentally: irreversible effects of chemicals will be additive upon repeated exposure and the resulting effect will be proportional to the total dose administered. As a consequence, a low concentration [c] administered for a long period of time [t] will produce essentially the same effect as a higher concentration administered for a shorter period of time (c x t = constant effect). By contrast, receptor-mediated processes will often be highly non-linear with quasi-thresholds at low concentrations and saturation behaviour in the high concentration range. Therefore, a weak or negligible response would be expected in the low concentration range for chemicals of this nature, even when present for long incubation periods. Analysis of the dose-time relationship of toxicity will therefore deliver important information on underlying mechanisms of toxicity. These considerations have direct consequences for the design of the experimental studies conducted within SEURAT-1, in that
An Adverse Outcome Pathway (AOP) describes the sequence of events between a molecular initiating event and an adverse outcome at the individual or population level (Ankley et al., 2010). Consequently, it is reasonable to categorise modes-of-action based on the chemicals’ reactivity, i.e. to make a distinction between compounds that show strong, irreversible interactions with intracellular targets from those that show weak, reversible interactions (Figure 3.1). Whereas the mechanisms related to highly reactive compounds are mostly well understood, those related to compounds showing weak interactions are often challenging. Hence, as a starting point, prototype AOPs for the development of in vitro test systems should be selected based on well-understood pathways, i.e. for highly reactive compounds, with later extension to compounds with low chemical reactivity (once their pathways are defined). This reasoning has been followed by the ‘Gold Compound’ Working Group in their strategy for selecting reference compounds for common use within SEURAT-1.

![Figure 3.1: Chemical reactivity triggers toxicological responses. Highly reactive compounds modify cellular targets permanently, while the low reactive compounds create reversible interactions.](image-url)
3.4 Rational Design of *in vitro* Test Systems

The SEURAT-1 strategy dictates that the general aim behind the design of *in vitro* test systems should be to capture at least one specific mode-of-action that is nominated *a priori*, in order to ultimately evaluate chemicals in some manner against that selected mode-of-action. However in pursuing this endeavour, test developers need to consider many other factors during the design, development and evaluation phases to ensure that their resulting test system is fit-for-purpose and can generate reliable data. Moreover, although there is an immense array of biological models, readout techniques and *in vitro* hardware platforms available to test developers to chose from, being surrounded by options regarding system components does not guarantee an efficient or ultimately successful outcome. What is needed therefore is a consistent, logical and transparent approach to test system design that helps to identify the most creative and feasible solutions while ensuring the best use of time and resources.

Toxicology by nature has a strong reverse-engineering character. In trying to understand why a substance causes an adverse effect in an organism, one must deconstruct the biological system and relevant processes and work backwards from outcome to cause. This is primarily a data-driven procedure involving the analysis and interpretation of observations (measurements) derived from toxicology experiments and related endpoints. Importantly, this reverse-engineering approach is fundamental to identifying and elucidating modes-of-action and related AOPs, to eventually build our knowledge-base of toxicology. However, the design and implementation of an *in vitro* test system that captures a specific mode-of-action for prospective toxicity assessment of chemicals is very much a direct-engineering problem which requires a very different approach. Here we explore such an approach and the typical steps involved.

The rational decision making model (Cross, 1989) is frequently employed by the engineering community to tackle complex design projects in an efficient and effective manner. It provides the basis for a formal and systematic process to progress from initial requirements right through to a final design specification. To enter the process therefore it is necessary to have first defined and sufficiently described the definite purpose of the system, together with a clear indication of what constitutes success and how to judge it. This may seem like an obvious prerequisite but in fact it is often the case that the purpose of an *in vitro* system is only defined after it has been evaluated in some generic manner. It is more akin to trying to answer the question, ‘what is this system good for now that I have it?’ as apposed to, ‘what do I need in a test system to achieve this purpose?’ Regarding purpose, a test system might for example be proposed to;
Determine the reactivity (molecular bioactivity) profile of chemicals to associate them with likely cellular-molecular targets, which in turn are linked to modes-of-action.

Capture a specific mode-of-action to discriminate chemicals that are likely or unlikely to induce toxicity in humans in that manner.

Identify chemicals that are associated with a specific mode-of-action and rank them with respect to their toxicodynamic potency.

Identify chemicals that are associated with a specific mode-of-action and indicate a quantitative point-of-departure that can be used to derive an in vivo effect-level, as a function of dose kinetics.

One of course could and should be more specific regarding the purpose of a test system but as illustrated in the examples above, it will usually be the case of addressing toxicological hazard identification or hazard characterisation, moving from a more qualitative assessment of toxicity to a more quantitative one, requiring increasing levels of functionality and performance from the system as the bar is raised. At this initial stage, it is also advisable to conceive of a validation strategy which would be suitable to determine if a test system actually meets expectations, i.e., is fit for purpose. Outlining the design of the eventual validation study before any design or method has been proposed ensures that the performance standards that are specified are as objective as possible and linked intrinsically with the defined purpose, rather than being subjectively linked to a specific test system. If at this point a validation strategy is not conceivable or specifiable, then it is likely that the purpose of the system lacks clarity or reason and therefore should be revisited. There is no point developing a test system that cannot be validated against a specific purpose since there will never be any evidence to demonstrate its reliability and relevance to end-users and decision makers.

Once the purpose of the test system and the validation strategy have been sufficiently defined, the rational decision making model can be employed to guide the design process. The output of the process is one or more final designs for fit-for-purpose systems together with a transparent and detailed description of how these designs were arrived at in a rational manner. The description of the design process is as important as the final design proposal itself since it provides confidence that investment of resources to embody the design in a prototype test system will be worthwhile, and if through validation the system is shown to fall short of expectation, weaknesses in the design can be rapidly identified and effectively addressed. The five key steps involved in the process are outlined in Figure 3.2. The first step is to compile an extensive list of attributes (properties, characteristics) that a test system should possess to achieve its purpose, ideally in a convenient and reliable manner. For example, such attributes may address the following aspects;
Biology/Toxicology

- capturing of one or more key events linked to one or more modes-of-action
- metabolic competence (both with regard to activation and inactivation)
- intactness of certain cellular metabolic and signalling pathways
- expression of certain receptors, transporters and cell type-specific properties
- capable of exhibiting particular biomarkers of effect
- demonstration of homeostasis during the entire observation period

Technicalities

- throughput of chemicals
- complexity of setup and operation
- time to result
- ease of cell culturing and differentiation
- need for specialist detection instrumentation
- use of readily available labware and reagents
- number of assays or elements in the system
- liquid handling requirements
- exposure and incubation requirements
- substrate requirement

Practicalities

- system and setup costs
- cost to test a chemical
- easy of transfer between labs
- accessibility by potential users
- level of operator training required
- time and cost to develop the system from design
- ease of validation
It is often useful to engage a wide group of scientists and technicians that are not specifically involved in the development project in order to harvest a wide range of unbiased views on what possible attributes a system should have to fulfil a particular purpose. Moreover, no attempt should be made to filter out attributes at this stage but to be as inclusive as possible. This ensures that potentially important design requirements are not inadvertently overlooked.

Once this attribute list is compiled, the next step is to apply a weight to each attribute that reflects its perceived importance for inclusion in the final design. It is often very beneficial to determine these weights by surveying a community of potential end-users and other test developers to again ensure a broad and impartial range of views. Once the relative weighting has been established it is possible to categorise attributes, namely, those that were consistently weighted high and thus can be considered as essential, those that received moderate weighting and thus can be considered as desirable but not necessarily essential, and those that received low weighting and thus can be considered as unimportant. At this point in the process the specification of system requirements, as reflected in the prioritised list of attributes, is complete.

Rather than immediately trying to conceive of a single design that satisfies requirements, the rational decision making model instructs that in fact the appropriate next step is to leave the attributes aside and start brainstorming designs based on a wide range of concepts, from the basic to the exotic. The aim is to be as creative as possible and not to prejudge design ideas before they have been sufficiently elaborated and described. Once this process has been completed, each design can be then assessed in an objective and systematic manner against the attribute list. Any design not possessing all the essential attributes is eliminated from further consideration, while those that do possess the essential attributes are then further prioritised based on how well they reflect the desirable attributes. As the concluding step, one or more selected designs are taken forward to be finalised and described in preparation for system prototyping and evaluation.

**Figure 3.2:** A summary of the key steps to follow for the rational design of a test system that should be fit for a particular purpose defined a priori.
Incorporating this design approach into the system development process within SEURAT-1 will take some effort since it reflects typical practice within the engineering world not often encountered by biologists and in vitro toxicologists (the synthetic biology area being an exception). However, since implementing the SEURAT-1 strategy relies heavily on a purpose-driven approach to test system design, development and validation, it is of utmost importance that test developers give serious consideration to adopting the rational design making model to guide their efforts.

3.5 Setting Goals and Achieving Them

The SEURAT vision and strategy provide the SEURAT-1 Research Initiative with a common direction and overall purpose. To complement this broader context and set clear goals at the cluster level, it has also been important to define SEURAT-1 objectives, as summarised in Figure 3.3. The first objective stems directly from the strategy and is intended to make the adoption of a mode-of-action approach explicit in the conception and execution of research activities. The second objective essentially captures project tasks aimed at producing a wide range of novel tools and approaches useful for safety assessment, while the third objective addresses the desire to combine this output to demonstrate added-value and impact. The forth and final objective has been defined to ensure that effort is invested to map out how the research approach and platform established during SEURAT-1 can be expanded in a continuous and targeted manner to influence and support concurrent and future research programmes, towards the eventual realisation of the vision.

**SEURAT-1 objectives**

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

*Figure 3.3: The SEURAT-1 cluster level objectives*
As clearly stated at the outset of the SEURAT-1 Research Initiative, and now reflected in the objectives, a major goal of the cluster is to demonstrate proof-of-concept in relation to key areas and deliverables. The expectation is that no individual project could manage to do this on its own, but that proving selected concepts will require the collective input from many projects. As summarised in Figure 3.4, it has been decided to stratify the concepts to be proven into three distinct levels, namely, theoretical, systems and application. Proof-of-concept at the theoretical level aims to show how toxicological knowledge concerning mode-of-action can be mined or perhaps generated, and then reconciled, consolidated and explicitly described in a format that can be managed and communicated in an effective and harmonised manner. Proving this concept will require not only acquiring and managing mode-of-action knowledge, but also the demonstration of how this knowledge has been used in a purposeful manner to drive the more applied research activities.

**Figure 3.4:** Addressing the proof-of-concept objective within SEURAT-1 will involve operating at three conceptual levels – theoretical, systems and application.

At the systems level, the intention is to demonstrate how test systems can be produced by integrating various *in vitro* and *in silico* tools emanating from the projects, in order to assess the toxicological properties of chemicals using mode-of-action as an analytical basis. Such systems may include for example, a combination of computational chemistry models with a battery of *in vitro* assays to generate a mixed set of chemical-structure and bioactivity descriptors that can be used to group chemicals into mode-of-action based categories, or the combination of biokinetics (PBBK) models with *in vitro* concentration-response assays to estimate *in vivo* no-effect levels in rodents and humans. At the highest level, proof-of-concept will address the desire to show how the data and information derived from the tools and methods developed within the cluster can actually be used in specific safety assessment frameworks and scenarios. These may include, for example, establishing thresholds of
toxicological concern derived from *in vitro* assays or how to use mechanistic data to improve the robustness and broaden the applicability of read-across.

The proof-of-concept objective adds a new dimension of deliverable at the cluster level, distinctly different from the deliverables anticipated at project level. The establishment of the SEURAT-1 Working Groups provide a horizontal dimension to complement the vertical dimension represented by the projects. The cross-project aspect of the Working Groups not only serves to stimulate and sustain project interactions and knowledge sharing, but can also be exploited to directly address the challenges posed by the proof-of-concept objective. Regarding proof-of-concept at the theoretical and application levels, the Mode-of-Action and Safety Assessment Working Groups are likely to take the respective leads, whereas proof-of-concept at the systems level will rely on yet another (third) dimension of cluster interaction, namely the interaction of Working Groups.

Setting objectives and goals is one thing, but achieving them is another. At the project level, the coordination and management functions together with the related work packages ensure that progress is tracked and that output is ultimately delivered as foreseen. At the cluster level too, progress has also to be monitored with respect to the achievement of the SEURAT-1 objectives outlined above. This task falls to the SEURAT-1 Scientific Expert Panel (SEP) which is in the ideal position to take a higher level, collective view on the evolution of the cluster work programme and to suggest corrective action and new initiatives if and when required. To facilitate this role and put it on a more formal footing, the SEP supported by COACH have committed to undertake a *Strategic Review* of the cluster on roughly an annual basis, the first taking place in June 2012. The motivation underpinning this Strategic Review is broad ranging, as summarised in *Figure 3.5*.

**Strategic Review - Motivation**

- Ensure that cluster-level objectives are achieved.
- Facilitate 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.

*Figure 3.5: Summary of the motivation underpinning the Strategic Review process to be undertaken by the SEP.*
The Strategic Review will utilise a number of basic project and organisational management tools such as PERT-like diagrams to map project interactions and a SWOT (Strengths, Weaknesses, Opportunities, Threats) analysis to obtain a collective view on the status quo. Identifying key deliverables from one project that can be channelled into other projects to enhance the work programme and accelerate progress will be of particular interest. The objectives and focus of the Working Groups will also be considered carefully during the review since they offer an extra capacity of the cluster to tackle common research questions and look for synergies between the projects that go beyond just the intersection of project work programmes.

In summary, significant inroads have been made within the SEURAT-1 Research Initiative to embrace and implement the mode-of-action inspired strategy, exemplified early on by the approach employed to select reference chemicals. Influence of the strategy is also being felt in the design and development of *in vitro* test systems where the purpose of such systems is intrinsically linked to assessing chemicals within a mode-of-action framework. The elaboration of SEURAT-1 objectives and the establishment of a Strategic Review process will not only facilitate effective progress monitoring at the cluster level, but will also allow the definition of an overall roadmap for charting the evolution of the research programme towards the achievement of SEURAT-1 goals.
References:


“The secret of getting ahead is getting started. The secret of getting started is breaking your complex, overwhelming tasks into small manageable tasks, and then starting on the first one.”

Mark Twain
4.1 Introduction

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

The following six 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 biological 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 coordinating 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. This second volume now contains the first results from the research conducted within the SEURAT-1 Research Initiative. It presents the research highlights and, thus, is not meant for providing a complete overview about all of the project activities. Each project description is amended with the following subchapters: (i) the innovative aspects with respect to the achieved results, (ii) the established cooperation with other projects of the SEURAT-1 Research Initiative, (iii) the expected progress within the second year of the project, (iv) future perspectives in the long run, describing possible next steps based on the achieved and expected results from the various projects. The overview about the Principal Investigators from each institution, organised within the projects completes these sub-chapters.

The detailed project descriptions are followed by a sub-chapter summarising the main activities within each of the projects as well as on the cluster level, including extended abstracts from the awardees of a poster session organised at the second Annual Meeting of the SEURAT-1 Research Initiative. Overall, this sub-chapter provides the transition from the level of the various projects to the cluster level and, consequently, is followed by a report about the cross-cluster cooperation. Here, we describe the modus operandi of cross-cluster Working Groups as the central elements for facilitating the cooperation between projects and people. In total, six Working Groups were established, which are: (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 project members from different projects, enabling targeted discussions on the needs and contributions of the SEURAT-1 research projects to meet the cluster-level objectives. Finally, a report describing the outreach activities finishes this chapter. The central aspects here are the organisation of the first SERAT-1 summer school, workshop activities and the SEURAT-1 public website. Besides the Annual Report, these are the most important cluster-level tools to promote the dissemination of knowledge.
4.2 SCR&Tox: Stem Cells for Relevant efficient extended and normalised TOXicology

The Need for Harmonised Quality Controls in Pluripotent Stem Cell-Based Toxicology Studies

Francesca Pistollato, Susanne Bremer-Hoffmann, Glyn Stacey, Christian Pinset, Vania Rosas, Marc Peschanski

4.2.1 Introduction and Objectives

In the last years various cellular models have been widely used for the development and validation of reliable and relevant *in vitro* toxicity tests, in the effort to optimise safety assessments of xenobiotics and at the same time to reduce and replace currently used traditional animal-based experiments. Amongst these, pluripotent stem cells (PSCs) have been judged as particularly suitable and have become an attractive alternative to the use of other human cell cultures, such as primary cells, which are difficult to standardise, or cell cultures with carcinogenic origin, which have often unwanted or unknown characteristics that might impact the cellular response to xenobiotics (Wobus et al., 2011). Both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) can be driven to differentiate towards any somatic cell type that can be targeted by hazardous chemicals. The use of stem cell derivatives offers the unique opportunity to create toxicologically relevant cellular model that can support the paradigm shift towards a mechanistically oriented safety assessment.

Nevertheless, the culture and differentiation of human PSCs are technically challenging and some important issues related to their use need to be addressed before they are ready for routine applications. Among these challenges are issues such as the stable culture of PSC lines, obtaining homogenous differentiated cell cultures and scale-up of the cells of interest in order to make sufficient numbers of high quality cells available for toxicological investigations. Given the peculiar nature of PSCs, a high level of standardisation of undifferentiated cell cultures as
well as of the differentiation process is required in order to ensure the establishment of robust and relevant test systems. It is therefore of pivotal importance to define and internationally agree on crucial parameters to judge the quality of the cellular models before enrolling them for toxicity testing. Taking into account past Good Cell Culture Practice (GCCP) guidance (Coecke et al., 2005), it is of great importance to foster a consensus amongst researchers using PSC lines for toxicological applications. The promotion of effective quality control systems and the delivery of sufficiently robust protocols enable the transfer of stem cell based test methods from one laboratory to another without facing the problem of significant inter-laboratory variability in toxicological data. Furthermore, the carefully defined quality standards based on the genotypic, phenotypic and functional characteristics of the cells will support the evaluation of the relevance of the cellular model used to answer particular toxicological questions. A further purpose of this objective within the SEURAT-1 Research Initiative is the facilitation of education and training, supporting smooth implementation of stem cell based toxicity testing in routine laboratory use.

It is important to mention that efficient preparation of highly purified, quality controlled PSC derived “tissues” might be difficult for laboratories involved in drug testing, thus many groups are currently relying on purchasing stem cells and their derivatives from distribution centres. Nevertheless, the evaluation of commercially available PSC derivatives for toxicological applications in the different industrial sectors, including the pharma, remains widely unsolved. New technologies based on single cell analysis might be applicable solutions to this challenge (Schroeder, 2011), even though significant research activities are necessary to apply these techniques on a routine basis.

One of the main objectives of the SCR&Tox project is the development of quality control (QC) standards that can be applied in routine PSC-based toxicity testing in order to:

- judge the toxicological relevance of data derived from stem cell based toxicity tests,
- monitor crucial culture steps, such as in differentiation protocols that will impact on the reliability of the data,
- provide guidance for non stem cell in vitro toxicologists in the use of these sophisticated cellular models.

The establishment of QC standards will be achieved in a step-based approach:

- the SCR&Tox partners (“Joint Research Centre” and “Health Protection Agency”) compiled and published a review manuscript on markers and QCs that are currently proposed in the scientific literature (Pistollato et al., 2012).
Additionally the same groups prepared and distributed a questionnaire on possible exploitable QCs for PSC-based toxicity testing to all SCR&Tox partners in order to obtain an exact overview of currently applied QCs.

It is planned to harmonise the QC standards for undifferentiated PSCs as well as for the various cell types within the SCR&Tox consortium but most probably also within the other projects of the SEURAT-1 Research Initiative ensuring the comparability of toxicological data.

The creation of common templates will allow a standardised data collection and submission and storage in a database of ToxBank

The responses to this consultation may enable quantitative thresholds to be set.

4.2.2 State of the Art in the Application of Pluripotent Stem Cells for Toxicity Testing

Pluripotent stem cells: definitions, characterisation and critical issues

Human embryonic stem cells (hESCs) are isolated from the inner cell mass of a human blastocyst between day 5 and 6 of development, a process that raises significant ethical issues in some countries. These cell lines theoretically have the potential of unlimited self-renewal whilst maintaining a stable genotype and phenotype. In addition, they retain the potential to differentiate into most cell types of the human body, a characteristic that is defined as pluripotency.

Induced pluripotent stem cells (iPSCs) can be derived from a variety of non-pluripotent cell types by artificially inducing the over-expression of specific genes (Park et al., 2008) involved in pluripotency. This important advance has opened up the possibility to generate PSCs from any individual, potentially leading to patient specific cell therapies and in theory eliminating the risk of graft-versus-host disease and immune rejection. iPSC technology also alleviates the constraints of a limited donor pool and the ethical issues associated with embryo-derived cell lines. An iPSC line is typically derived by transfection of certain genes for stem cell-associated molecules, transcription regulators (e.g. Oct-3/4 (Pou5f1), Sox2) and other genes that enhance efficiency of induction (e.g. Klf4, c-myc), into somatic (i.e. non-pluripotent) cells, such as adult fibroblasts. Transfection and expression of the genes is typically achieved by viral vectors, such as retroviruses and lentiviruses. After 3-4 weeks, small numbers of transfected cells give rise to colonies of PSCs that may be isolated through phenotypic or reporter gene/antibiotic selection. Multiple lines of evidence suggest that there is a remarkable similarity between hiPSCs and hESCs in their morphology, growth properties, stem cell markers expression, telomerase activity and pluripotency (Krueger et al., 2010). For these reasons, SCR&Tox normally uses hESCs as a gold standard cell type when setting up

1 - http://mbbnet.umn.edu/scmap.html
hiPSCs-based toxicity studies. Nevertheless, it is important to underline that there might be subtle functional differences between directed differentiation of hiPSCs and hESCs (Daniels et al., 2010). Additionally, the persistent expression of donor cell genes among hiPSCs seems to contribute to differences with hESCs (Ghosh et al., 2010). These data need to be taken into account when assessing the relevance of hiPSCs in relation to natural pluripotent hESCs for toxicity testing.

Particularly, the undifferentiated state of initial PSC culture is a critical aspect to consider, as the differentiation process, the phenotype or functionality of the differentiated cells might be affected, possibly compromising the results of a toxicity test. To characterize undifferentiated hESC and hiPSCs cultures, molecular analysis, typically by quantitative PCR (qPCR) for expression of a limited number of key pluripotency related genes, such as Nanog, TDGF, Oct4, GABRB3, GDF3, DNMT3 and PODXL, is required (Adewumi et al., 2007). In addition, lineage specific markers such as AFP (expressed in the endoderm), brachyury (expressed in the mesoderm) and Sox-1 (expressed in the ectoderm), should yield negative results by q-PCR (De Miguel et al., 2010). Epigenetic markers of early development should also be considered when initially characterising hESC and hiPSC cells. For hiPSCs characterisation, the level of expression of DNMT3B has been proposed as an indicator of complete cell reprogramming (Chan et al., 2009).

Importantly, users of PSCs should follow culture protocols recommended by the supplier of the cell lines and any alteration to the protocols will require thorough documentation and qualification using a set of standard criteria (Adler et al., 2007). Both hESCs and hiPSCs grow efficiently on feeder cells, generally mouse embryonic fibroblast or human neonatal fibroblasts (Williams et al., 1988). Batch testing of critical reagents subject to greatest biological variation, including feeder cells, is mandatory from a scientific perspective. It will be important to adopt feeder-free systems to eliminate the feeder cells which are one of the most variable and ill-defined components in PSC culture systems.

The culture and expansion of both hESCs and hiPSCs are challenging for a number of reasons including that: (i) they grow as colonies generally difficult to culture, (ii) the most commonly used method for passaging colonies is micro-dissection and re-growth from small colony fragments (i.e. “cut-and-paste”) on feeder cell layers, (iii) there are critical factors involved in preserving the undifferentiated state (e.g. feeder cells, media, serum replacement and growth factors) and these are not yet fully understood, (iv) undirected differentiation occurs persistently under standard culture conditions and this cannot yet be fully controlled.

Pluripotent stem cell-derived cultures for toxicology testing

A major challenge for the application of PSCs in toxicology and drug discovery is to demonstrate that they can reproducibly generate a significant range of toxicologically relevant cell types
and that these provide cell preparations with characteristics and responses typical of human tissue types. A further goal would be to obtain data from stem cell based systems that can indicate chronic toxicity effects (e.g. steatosis, cholestasis, cardiomyopathies, etc.) as well as acute responses, such as apoptosis, necrosis etc. Nowadays, several differentiated cell types derived from both hESCs and hiPSCs have been judged to have potential utility for toxicology studies, mainly for neurotoxicity, cardiotoxicity and hepatotoxicity but also for reproductive and developmental toxicity (Pistollato et al., 2012). However, an international agreement on the level of cellular differentiation of PSC-derivatives is required for certain toxicological questions before further evaluating and validating stem cell based toxicity tests. As discussed above, differentiated cell derivatives obtained from both hESCs and hiPSCs are often heterogeneous and various techniques have been proposed to select specific cell types relevant for toxicity testing. Those most commonly applied are use of selective culture conditions, selection on the basis of cell surface markers by flow cytometry and fluorescence activated cell sorting (FACS) and use of antibodies bound to magnetic beads. Genetic selection may also be based on the development of genetically modified hESCs which express a reporter/ selectable marker under the control of regulatory elements of genes expressed in specific cells.

In order to establish the suitability of a specific differentiated cell type for toxicology studies, the differentiated cell population should be shown to express at an appropriate level specific differentiation related markers. In the case of neural cell systems candidate antigens might include β-III-tubulin, MAP2, neurofilament 200 (NF200) and Synapsin-I for mature neurons, whilst for cardiomyocyte derivatives, brachyury, alpha-cardiac actin, atrial natriuretic factor and the specific sarcomeric myosin heavy chain (clone MF20). Once a marker panel has been selected, specific quality control (QC) methods are needed to establish acceptability criteria, which, importantly, should also include cell functionality (Pistollato et al., 2012). For example, PSCs-derived neurons should be proven to be electro-physiologically active, generating action potentials. In this case, specific QC metrics for the functional activity and threshold levels for a positive phenotype need to be defined in order to properly judge the suitability of an individual cell preparation for use in a toxicology assay. In general, a well-defined set of QC analyses should serve as basis for acceptance criteria supporting a reduction of intra- and inter-laboratory variability of the test system and this is also a prerequisite for novel toxicological in vitro tests based on PSCs.

### 4.2.3 Approach: Threshold Values for QC Standards

Revision of existing QC standards for PSCs and harmonization of QCs for PSC-based toxicity testing within the SEURAT-I cluster

In order to define possible QC assays to be exploited for PSC-based toxicity testing, two SCR&Tox partners (“Joint Research Centre” and “Health Protection Agency”) have compiled
and recently published a review manuscript on markers and QCs that are currently proposed in the scientific literature (Pistollato et al., 2012). It is envisaged that QCs standards for PSCs-based toxicity testing will be harmonized within the SEURAT-1 Research Initiative. In order to develop reliable and relevant tests that can serve in toxicity testing, the SCR&Tox partners “Joint Research Centre” and “Health Protection Agency” proposed a collection of QC assessments to the other SCR&Tox partners in the format of a template. This template can also be wider used within the whole SEURAT-1 Research Initiative as proposed during the 2nd annual meeting in the focused session on stem cells (see chapter 4.9.2 and 4.10.6). The collection of data derived from agreed QC standards might serve as a basis for a SCR&Tox guidance document that will be developed by the end of the project. The discussion on harmonized QCs had been further expanded within the SEURAT-1 Research Initiative.

Selected QC standards for PSCs applied in the JRC

In the effort to provide potential threshold values relative to the selected QCs, SCR&Tox partner “Joint Research Centre” selected a panel of the most critical QCs which are currently applied on a hESC cell line (H9, from WiCell) and two hiPSC lines: IMR90 and 4603 (received from SCR&Tox partner I-Stem, both wild type). The main aim was to create a template of possible QC standards, with the relative preliminary thresholds, to be used for the characterization of undifferentiated PSCs and their differentiated neuronal derivatives.

4.2.4 Results: Quality Control Assays

QC Assays applied at “Joint Research Centre” for the characterization of undifferentiated PSCs

As mentioned above, the difficulty of maintaining a high proportion of undifferentiated hESC and hiPSCs in routine cultures should not be under-estimated. Cultures may quickly become largely composed of differentiated cells and this will be visible both by the appearance of morphologically altered cells and colonies and by changes in marker expression. Morphological evaluation of cell colonies should be carried out daily and representative colonies should be documented periodically using digital photography (Adler et al., 2007). Additionally, undifferentiated PSC cultures should be positive for a panel of key markers (Conley, 2004; Kolle et al., 2009).

In order to evaluate the undifferentiated and pluripotent phenotype of available PSCs, SCR&Tox partner “Joint Research Centre” routinely performed some critical QC analyses: daily analysis of cell/colonies morphology, analysis of alkaline phosphatase activity (normally present in PSCs), qPCR analyses of pluripotency related genes, analysis of pluripotency related marker expression by both flow cytometry and immunocytochemistry, followed by high content imaging. The following images and descriptions give a fair representation of how
these crucial parameters should be analysed, together with their relative expected results, both at a qualitative and quantitative level. These analyses have been done on two PSC lines: the hESC line H9 (WiCell) and the wild type hiPSC line IMR90 (generated SCR&Tox partner I-Stem). When setting up QC parameters for hiPSCs, it is important to use also hESCs as a comparative gold standard, at least during the preliminary phases.

According to the applied QC assays, both hESCs and hiPSCs should be round in shape, with large nucleoli, a small nuclear: cytoplasmic ratio and at least 85-90% of colonies should be flat and tightly-packed, which is a typical morphology of an undifferentiated cell colony (Figure 4.1A, B). More than 80% of the colonies should present alkaline phosphatase activity (Figure 4.1C, D). Accordingly, analyses of qPCR for pluripotency related genes (Nanog, Oct4 and Sox2) and of ectodermal related genes (Sox1 and nestin) should show low expression of ectoderm related genes and high expression of pluripotency related genes (Figure 4.1E).

**Figure 4.1** Colonies morphology, alkaline phosphatase and qPCR analyses. (A, B) Representative phase-bright images of undifferentiated colonies; (C, D) representative images of alkaline phosphatase stained colonies; (E) Bar graph reporting qPCR analyses of Nanog, Oct4, Sox2, Sox1 and Nestin, normalized to B-actin and GAPDH and then calibrated to undifferentiated H9 cells (ΔΔCt method). Mean of 3 independent analyses ±S.E.M.

Quantification of the cell subpopulations, typically by immunocytochemistry and flow cytometry, is essential to provide information on the proportion of undifferentiated versus differentiated cells. PSCs should result to express SSEA3, Oct4, Tra1-60 and TRA1-81 in at least more than 80% of the colonies (Figure 4.2A-F) both by immunocytochemistry and flow cytometry (Figure 4.2G) and these results should be reproducible over passages.
In addition, hESC and hiPSC cultures should be subjected to molecular analysis, typically using qPCR, to determine the level of expression of a limited number of key pluripotency related genes (Adewumi et al., 2007). To assess stem cell pluripotency, JRC used the common approach based on “spontaneous” embryoid bodies formation (Figure 4.3A, B), which can form the three germ layers. Analyses of some germ layers specific genes should indicate a highly significant increase of endoderm (AFP, KRT18), ectoderm (Nestin, Sox1 and Pax6) and mesoderm (NPPA and Brachyury-T) related gene expression (Figure 4.3C, D). According to these results, JRC formulated preliminary threshold values for the analyzed QCs.
Figure 4.3 Embryonic bodies (EBs) formation assay and analyses of 3-germ layer related genes. (A, B) Representative phase-bright images of embryonic bodies at day 1; (C, D) Bar graphs reporting qPCR analyses of AFP, KRT18, Nestin, Sox1, Pax6, NPPA and Brachyury-T, in H9 (C) and IMR90-derived embryonic bodies (D), normalized to B-actin and GAPDH and then calibrated to their own undifferentiated control (day 0) (ΔΔCt method), mean of 5 independent analyses ±S.E.M. Statistical analyses have been done by using 1-way-Anova, Newman-Keuls post-test (*** p≤0.001; ** p≤0.01; * p≤0.05).

QC Assays applied at at “Joint Research Centre” for the characterization of PSC derived neuronal cells.

PSC derived cell populations should express at an appropriate level specific differentiation related markers, which, in the case of neural cell candidate antigens, might include β-III-tubulin, MAP2, NF200 and Synapsin-I for mature neurons. Once a marker panel has been selected, specific QC methods are needed to establish acceptability criteria, which should also include cell functionality (Pistollato et al., 2012). Indeed, PSCs-derived neurons should be proven to be electro-physiologically active, generating action potentials. As a consequence, specific quality control metrics for the functional activity and threshold levels for a positive phenotype need to be defined in order to properly judge the suitability of an individual cell preparation for their use in a toxicology assay. For the characterization of PSC-derived cells,
qPCR analyses, immunocytochemistry followed by high content imaging and multi-electrode array (MEA) analyses have been performed at SCR&Tox partner “Joint Research Centre”.

The following images qualitatively and quantitatively describe some selected crucial QC parameters which might be used for the characterization of PSC derived neuronal cells suitable for neuro-toxicity testing. Again, also for these analyses hESC differentiated cultures have been used as comparative gold standard.

PSC derived neuronal cells should show a significant decrease of pluripotency-related marker expression (Oct4, SSEA3 and Tra1-60) upon differentiation (in this case after 28 days of differentiation), parallel to a decrease in the number of Ki67+ cells, a very well described cell cycle marker (Figure 4.4B, C) which hints to the postmitotic phase of the neuronal cell culture. On the contrary, the proportion of neuronal cells (B-III-Tubulin+, MAP2+, NF68+ and NF200+ cells) should significantly increase (Figure 4.4A-C). Also, the presence, the number and the length of neurites generated in differentiated cell cultures might be verified (graphs not shown).

According to the immunocytochemistry data, qPCR analyses should indicate a significant downregulation of pluripotency related genes (Oct4 and Nanog) in neuronal differentiated cells; conversely, neuroectodermal related genes (Pax6, Sox1 and nestin) should be generally upregulated compared to undifferentiated cells and a marked increase in the expression levels of neuronal related genes (NCAM1 and MAP2) should be recorded (Figure 4.4D).
**Figure 4.4** Analysis of pluripotency and neuronal related markers in PSC-derived neuronal cells. (A) Representative pictures of NF200 staining. (B, C) Bar graphs reporting mean average intensity of pluripotency related markers, of the cell cycle marker Ki67 and of neuronal related markers. Mean ± S.E.M. of 5 independent analyses (unpaired t-test, one-tailed). (D) Graphs reporting qPCR analyses of Nanog, Oct4, Pax6, Sox1, Nestin, NCAM1 and MAP2, all normalized to B-actin and GAPDH and then calibrated to undifferentiated cells (ΔΔCt method). Mean ±S.E.M. of 6 independent analyses for IMR90 and of 4 independent analyses for H9.

Additionally, analyses of specific neuronal subtypes should be carried out to evaluate neuronal cell culture heterogeneity (Figure 4.5).
Figure 4.5 Immunocytochemistry images of PSC-derived neuronal cell types. (A) Representative immunocytochemistry images of GABA (for GABAergic neurons), TH (for dopaminergic and noradrenergic neurons), VGluT1 (for glutamatergic neurons) and Isl-1 (for motor neurons).

Importantly, in order to verify the functionality of differentiated cell cultures, the generation of extracellular electrical activity by doing electrophysiological measurements with the MEA system should be verified. PSC-derived neurons should generate action potentials over a minimum firing rate (i.e. number of spikes/min), which we established to be ≥ 30 spikes/min (Figure 4.6).

Figure 4.6 Multielectrode array (MEA) system and MEA analyses of PSC-derived neuronal cells. (A) Image of a MEA chip and (B) image of the complete MEA system (available in the Systems Toxicology Unit of SCR&Tox partner “Joint Research Centre”). (C) Representative phase bright images of IMR90-derived neuronal cells cultured on a MEA chip (left), and report of mean firing rate (i.e. number of spikes/min).
4.2.5 **Innovation**

Complete characterization of cellular models is a prerequisite for their use in *in vitro* toxicological assays including stem cell based toxicity tests. However, the unique nature of PSCs requires the establishment of critical control standards assessing their pluripotency, as well as their successful differentiation into toxicologically relevant target cells. A variety of markers based on different technologies and functional readouts have been proposed to evaluate the pluripotent status of stem cells as well as to characterize the functionality of their cell derivatives. Emerging technologies such as ‘-omics’ will expand the knowledge on the PSCs profiles but will also support the identification of molecular mechanisms relevant for toxicity assessments. Nevertheless, it is a balancing act to identify markers and their level of expression that provide sufficient confidence on the pluripotent status of the PSCs as well as on the phenotypic identity/cell functionality of their derivatives and their applicability in routine toxicological laboratories. A pragmatic approach should be favoured in order to support a wide implementation in the use of stem cells for safety assessments.

4.2.6 **Cross-Cluster Cooperation**

The *SCR&Tox* consortium is a transversal partner benefiting to and from each of the other consortia all along the *SEURAT-1* Research Initiative. Because of this positioning, *SCR&Tox* partners collectively encompass a wide array of methodological and technological interests that allows us to identify partners whose interests are at the interface between *SCR&Tox* and each of the other consortia from the *SEURAT-1* Research Initiative, allowing permanent expert linkage. Our “Karolinska Institute” partner collaborates within NOTOX; the “Joint Research Centre” partner is actively participating within the DETECTIVE programme and the “Health Protection Agency” partner participates within the ToxBank consortium favouring collaboration.

*SCR&Tox* leads one of the four *SEURAT-1* working groups on Stem Cell Standardization and Characterization, whose first meeting was held in conjunction with the 2nd *SEURAT-1* annual meeting, and that is summarized in another chapter of this book (see chapter 4.10.6).

Also, the *SCR&Tox* proposal is to define at the earliest time point the main collaborative framework and cross-match specific programmes, in particular, to prepare (choosing and designing) at mid-term the most interesting assay for one toxicity pathway on which particular focus will be placed in the second half of the programme. This will be done to search for actual proofs of concept for each project of the *SEURAT-1* Research Initiative and, at the end of the 5 year-long programme, will allow defining precisely the advancement obtained and helping design the next objectives. It is clearly acknowledged that the *SEURAT-1* Research Initiative is a first step on a path towards the paradigm shift expected in toxicology, which will require long-term research investments and coherent activities. *SCR&Tox* has made a proposal to all
SEURAT-1 project coordinators to start discussing on the choice and design of the cell-based assay for toxicology testing in a joint effort to a successful common trial.

4.2.7 Expected Progress within the Second Year

The successful outcome of the SCR&Tox project will be gauged from the advances in our capacities to: (i) produce the relevant biological resources in needed quantity and quality for us, as well as for the other consortia, in the SEURAT-1 Research Initiative, (ii) design and implement methodologies proposed by others in the SEURAT-1 Research Initiative for exploring cell function and (iii) put them into practice for developing assays based upon the identification of biomarkers linked to toxicity pathways by other consortia in the SEURAT-1 Research Initiative, in order to (iv) bring the proof of concept of the use of pluripotent stem cells derivatives in the new paradigm of toxicology testing by demonstrating their value at the required scale on industrial platforms. These will be measured first and foremost through the final demonstration of at least one prototype high throughput assay for toxicology testing susceptible to enter the normalization and validation process, the end result of the articulated work of all consortia in the SEURAT-1 Research Initiative.

For the second year of the SCR&Tox project we will:

- Continue working on the amplification and banking of all quality controlled undifferentiated stem cells
- Continue working on the identification of robust protocols for differentiating pluripotent cells into derivatives –both at a “full” terminal stage and in applicable cases at an intermediate, amplifiable stage- for each of 5 toxicological relevant lineages (Liver, CNS, Heart, Skin, and Muscle).
- In parallel, we will continue working on the implementation of technologies for: cell engineering, defining cell profiles of gene and protein expression and exploring cell functions at the gene, protein and electrogenic levels.

4.2.8 Future Perspectives

The capacity to generate and differentiate patient-derived induced pluripotent stem cells (iPSC) into many relevant cell types, and their amenability to genetic engineering, opens the perspective of systematic industrial iPSC banking and differentiation to provide cells or tissues recapitulating human genetic diversity, physiology and pathology. However, it may be difficult to recapitulate the phenotype of complex and multifactorial diseases or toxic responses in isolated cells. To solve this problem, a "genome-based combinatorial approach for drug discovery and predictive toxicology testing" could be envisaged. This new paradigm relies on four main attributes of iPSC that make them a most promising tool:
Their indefinite self-renewal capacity at the undifferentiated stage, allowing provision of any amount of cells with a common genetic background, as well as consistency in biological material.

Their pluripotency. Depending upon the identification of appropriate and robust protocols, this allows differentiation into any cell phenotype of interest with a common genetic background.

Their potential at expressing any human genomic background. Given that suitable donors are available, iPSC lines can provide a cell model for any human genotype.

Their amenability to genetic engineering, allowing the generation of discrete models of gain or loss of gene function in any cell phenotype with an otherwise common genetic background.

These properties have already permitted both the successful identification of molecular mechanisms associated to monogenic diseases in iPSC progeny and ongoing studies aiming at using these in vitro models for high throughput screening in drug discovery to identify safe drug candidates. This approach will open new paths for predictive toxicology. We hypothesize that toxic responses in target organs from patients are different from that in healthy individuals, and thus that safety testing of new drugs should be fitted to iPSC lines of the relevant clinical population. Large banks of iPSC lines derived from randomly sampled specific patient groups, and from supposedly healthy people as a reference, both also representative of human genetic diversity, will allow us to establish predictive target-population specific toxicology screens to challenge drugs still at a pre-clinical development stage. Emphasis will be on the development of relevant 3D models using an appropriate combination of cells mimicking the in vivo toxicity. Such molecular screens could be used for direct comparison of toxicology profiles, benchmarking drug candidates with existing molecules and enable a “phase III study in a dish”. Furthermore, where some drugs are toxic to certain patient subpopulations which may be due to their (epi-)genetic “polymorphisms”, iPSC cell lines provide a basis for “population scale” analyses seeking discrete polymorphisms involved in an observed toxic phenomenon and, by extension, the molecular pathways that may be affected by the change in gene expression or function related to those polymorphisms. Most importantly, that knowledge of affected molecular pathways may lead to novel toxicity testing strategies and assays. We thus envisage a genome-based combinatorial approach for predictive toxicity involving stratified cohorts of patients treated with the same compounds but which exhibit differential toxicity profiles. The genomic and epigenomic alterations critical for the toxicity will thus be identified and the pathways analyzed using transcriptomics and proteomics.

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

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

These strategies are summarised in Figure 4.7.
Figure 4.7 Outline of an innovative approach for harnessing pluripotent stem cells for toxicology.

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


**Patents of project members related to the field of research**


**Recent key references of project members (last 3 years) related to the field of research**


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4.3 HeMiBio: Hepatic Microfluidic Bioreactor

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4.3.1 Introduction and Objectives

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 1 month in vitro. Such Hepatic Microfluidic Bioreactor could serve to test the effects of repeated exposure to chemicals, including cosmetic ingredients. To create this device, the cellular components of the liver need to be viable for over 1 month, with in vivo-like metabolic and transport function, and physiology. The latter includes (i) flow through the device, (ii) zonation of the hepatocytes (and some non-parenchymal liver cells), and (iii) impact of the non-parenchymal cells on the function and downstream toxicity of hepatocytes. The device should be able to (iv) screen drug-drug interactions as well as long-term toxicity of chemical entities. Finally, (v) the effect of enzyme inducers and inhibitors on the function of the liver-simulating system should be testable. However, currently, no bioreactor has yet been created that can indeed fulfil all the above-listed criteria. 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 the cellular function maintenance.

To mimic the liver function, many increasingly more complex and clinically relevant approaches are currently being used. However, these approaches are not satisfactory due to the shortage of human livers, as well as the fact that primary hepatocytes rapidly de-differentiate under standard conditions. Hence, what is needed for the cosmetics and pharmaceutical industry are the innovative culture systems that incorporate hepatocytes as well as non-parenchymal liver cells, derived from expandable/renewable cell sources. HeMiBio seeks to address
this unmet need using human induced pluripotent stem cells (iPSC), which offer a unique opportunity, given their expected capacity to self-renew and differentiate efficiently into the desired cell type. As an alternative, the consortium will test whether cells isolated from livers can be expanded by genetic manipulation using the UpCyte® technology, without loss of mature cellular function. Finally, co-cultures generated by the consortium will allow induction and maintenance of mature hepatocyte, hepatic stellate cell (HSC) and hepatic sinusoidal endothelial cell (LSEC) function, while creating a bioreactor that can provide clinically relevant information on drug and chemical clearance and toxicity. This will allow testing of repeated dose toxicity for several weeks to, ultimately, months.

Figure 4.8 Schematic representation of a liver sinusoid (adapted from: Dollé et al., 2010).

The underlying hypothesis for the successful creation of a 3D liver-simulating device suitable to test repeated dose toxicity is that: (i) hepatocytes and non-parenchymal cells need to be combined; (ii) both homotypic and heterotypic cellular interactions between the different components are required to maintain the functional, differentiated and quiescent state of each cell component; (iii) (a) the matrix whereupon cells are maintained, (b) oxygenation, (c) nutrient transport will need to be optimised to support long-term maintenance of hepatocyte
and non-parenchymal cell function, in an environment where shear forces are kept at their in vivo-like levels; (iv) the system needs to be built such that repeated 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 4.8 may not be required, the very short distance cellular interactions shown between (A) hepatocytes-LSECs and (B) hepatocytes-HSCs 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.

The overall objectives to achieve the creation of a liver-bioreactor, taking into account the hypotheses stated above, were outlined in the first Annual Report of the SEURAT-1 Research Initiative (Verfaillie, 2011). In brief, they include the tool development in order to engineer the different liver cells to be used in the bioreactor, the use of different types of sensors (microsensors and molecular sensors), the development of the bioreactor, the assessment of the cellular components in the bioreactor over time and the proof-of-principle study that a liver-simulating device can recreate the toxicity profile of chemicals in vitro with a known in vivo toxicity profile over a minimum of one month. In this second Annual Report, we focus on the generation of the different bioreactor prototypes, including the incorporation of high-resolution fluorescent markers into pluripotent stem cells, as well as the initial complement of the electrochemical sensors. This reflects the progress made on the following, specific objectives:

- Development of tools able to detect subtle modifications in the cells themselves (molecular markers introduced in the different cellular components) or of the extracellular medium in the near vicinity of the cells and/or of the cell culture medium for periods of up to four weeks. More specifically, this includes:

  a) The Incorporation of molecular sensors into the different liver cells to dynamically measure cell function and toxicity in a high-throughput format. High-resolution fluorescent markers will be developed and integrated in the different cell components to detect early inflammatory/pro-apoptotic effects.

  b) The Integration of innovative electro-chemical and optical sensors in 3D-bioreactors to allow assessment of function (e.g. oxygen uptake, ammonium, and glucose concentrations), as well as the continuous assessment of cell integrity (e.g. by measurement of potassium, and enzyme release due to cell death).

- Development of a 2D-bioreactor for the efficient isolation of differentiated iPS cell mixtures by trapping different cell types on micropatterned surfaces. This platform will be used to evaluate the role of cell-cell and cell-matrix interactions in the maturation and maintenance of functional hepatocyte
and non-parenchymal cells. The platform will serve as a rapid intermediary to the 3D-bioreactor and be used to explore varying sensor designs and cell interactions needed in the more complex design (see the following objective).

**Generation of a 3D liver-simulating device** mimicking the human liver, which reproduces the function of the hepatocyte and non-parenchymal liver cells over one month in culture. This will be accomplished by combining the engineered cells and sensors under the conditions characterised in the previous objective. The liver-simulating device created in HeMiBio will thus allow for the dynamic monitoring of cellular function and health in a high-throughput format under numerous conditions.

### 4.3.2 Generation of Microsensors and Cells Containing Molecular Sensors

**State of the Art**

**Molecular sensors**: We will use zinc-finger-mediated homologous recombination (ZFN-HR) to insert selection cassettes downstream of either a gene expressed specifically in mature hepatocytes, HSC or LSEC, or cell damage-specific expression cassettes (NF-κB, p53 and caspase-3). This combination will allow precise detection of toxic effects on any of the three cell components to be incorporated in the bioreactor.

It has been shown that the most reliable method to achieve precise lineage-specific gene expression is to introduce selection and/or marker cassettes using a precise knock-in strategy. Traditionally, lineage-specific marker genes have been introduced into the host cell DNA in a random fashion, using either viral vectors or plasmid DNA. A random approach has several disadvantages, including (i) silencing of the inserted transgene over time (Liew et al., 2007), which is a well-known phenomenon in ES cells and iPS cells whereupon differentiation large portions of the host cell DNA become silenced; (ii) variegation of transgene expression (Panel & Ellis, 2001), with the risk that expression of the introduced marker does not faithfully represent endogenous gene expression; and (iii) insertional mutagenesis, such as the disruption or unregulated over-expression of endogenous genes vital for this study (Goessler et al., 2006; Hematti et al., 2004).

Two recent studies reported, however, that gene targeting in human iPS cells can be achieved at high frequency by using ZFN and cleverly designed targeting/selection cassettes (Hockemeyer et al., 2009; Zou et al., 2009). ZFN consists of a non-specific endonuclease domain linked to a specific DNA recognition domain, which is composed of zinc-finger motifs designed to bind a specific DNA sequence. Upon binding of the two ZFN subunits to a chosen recognition sequence in the human genome, the nuclease domains dimerise and insert a DNA
double-strand break (DSB) at the target site, which leads to rapid activation of the cellular DNA repair pathways. In the presence of a DNA template that shares homology with the sides flanking the DSB (Cathomen & Joung, 2008; Cathomen and Schambach, 2009), the template is used as a donor DNA to repair the DSB and, at the same time, genetically modify the target locus. Depending on the number of zinc-finger motifs contained in the DNA binding domains, a ZFN pair recognises a target sequence of 18-24 bp, which, theoretically, is long enough to specify a unique target sequence in the human genome.

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

On one side, optical detectors are commonly used due to the simplicity of the microfluidics-detector interface. Optical fibres in particular can be incorporated in microsystems without difficulty. They provide a universal sensing platform as they are easily integrated with a multitude of different sensing schemes. Such schemes enable the preparation of a multitude of sensors from relatively straightforward pH sensors, to more complex ones, including high-throughput cell-based arrays (Epstein & Walt, 2003). Furthermore, the recent development of optical-fibre biosensors that can be used in combination with different types of spectroscopic techniques, e.g. absorption, fluorescence, phosphorescence and surface plasmon resonance (SPR), make them an interesting option for cell functional monitoring (Bosch et al., 2007). Electrochemical detection systems offer good detection limits for various analytes of biological interest. Among them, biosensors provide an attractive means to analyse the content of a biological sample due to the direct conversion of a biological event to an electronic signal (Wolf, 2003). Microelectrodes are one of the main tools for measuring cellular electrophysiology, oxygen, nitric oxide, neurotransmitters, pH and other various ions.

The most implemented microsensors in cell-based systems are aimed at monitoring the physical conditions of the cellular microenvironment (temperature) and the chemical conditions (pO₂, pH) (Wolf, 2003). Such microsensors can also be used to monitor specific cellular functions and cell metabolism. A prototype for chemosensitivity testing was, for instance,
used to simultaneously analyse changes in extracellular acidification, oxygen consumption and electronic impedance in live liver tissue (Sprague et al., 2006). Cell death was also monitored and quantified in real-time using a microfluidic platform equipped with K⁺-selective microelectrodes, based on the measurement of potassium efflux (Generelli et al., 2008).

Approach

One of the major challenges in building a 3D-liver bioreactor is the lack of data on the complex environment present inside the bioreactor where the cells live, aggregate and differentiate. This is particularly true when the number of cells is low. Most of the time, physiological samples are aliquoted to determine hepatic functions and metabolism, which is an inaccurate, time-consuming and often destructive process, in particular, when the sample volumes are small. In these circumstances, microsensors located in the direct vicinity of the cells can play a vital role, by monitoring cell culture conditions and, thus, help mimic the natural microenvironment. Furthermore, such microsensors can also measure either in real-time or at specific interrogation times (for instance, after a toxic insult) relevant parameters of the state of the cells. Likewise, molecular sensors built into the cells themselves, can provide information not only on the state of the cells, such as differentiation to mature cell types, but also on toxic effects on specific cell types. Such information is crucial to study the current cell conditions and may even be more central as a predictive indicator for long-term toxicity, in particular in conjunction with repeated dose delivery.

Two complementary approaches are pursued in this activity: intracellular optical observation by means of reporter gene constructs and extracellular chemical sensing of secreted or excreted material. These approaches are allocated to separate tasks, which are described in more detail below.

Molecular sensors: In order to monitor in real-time the effect of toxins (cosmetic and pharmaceutical products) on the cellular components of the liver, it will be important to assess early stress/damage to cells. Therefore, we plan to introduce molecular sensors that allow us to assess NF-7 kB or p53 activation, or caspase-3 activity in the three cellular components to be incorporated in the bioreactor, hepatocytes, HSC and LSEC. The ultimate multiwell bioreactor would then allow detection of toxicity to specific cell population preceding toxicity to one of the other cellular components, something that is nearly impossible in vivo, or in bioreactors without the molecular sensors to be incorporated in liver cells that can be individually identified thanks to fluorescent probes incorporated behind lineage specific promoters (Figure 4.9).
Figure 4.9 Multiwell bioreactor containing cells equipped with molecular sensors. If a toxin causes apoptosis of HSEC first, the time course analysis would demonstrate that well 5-8 display co-fluorescence of GFP and cherryRed, whereas co-fluorescence of GFP and cherryRed in wells 9-12 and 1-4, as well as release of ALT (alanine transaminase) would follow in a second phase.

However, silencing or variegation after random integration are known obstacles to reliable transgene expression, especially in ES cells, in which transgene silencing after gene transfer due to the changes in chromatin state upon differentiation is a known phenomenon. One mechanism to overcome these problems is to introduce the reporter genes in regions of the host cell DNA that are unaffected by general changes in chromatin state, such as the AAVS1 (Adeno-associated virus integration site 1) locus, located on human chromosome 19, that encodes the ubiquitously expressed PPP1R12C gene (Hockemeyer et al., 2009). We propose to introduce a Flippase Recognition Target-sites flanked PGK-Puro\textsuperscript{\textregistered}-HSV-TK cassette into the locus, using established ZFN. Upon characterisation of a genetically modified master hiPS cell clone carrying this cassette, we will subsequently introduce the sensor cassette genes using the highly efficient Flippase recombinase system (Mortensen, 2007). It should be noted that once the Flippase Recognition Target sites are introduced in the AAVS1 site, within this locus, additional molecular sensors not described hereunder can be introduced, such as, for instance, sensors that would measure capillarisation of LSEC, or activation of HSC, among others.
Microsensors: Microsensor tools provide indispensable information on two aspects: firstly, they supply the input essentially needed for an active control, i.e. the external regulation of culture conditions, such as physiological medium composition. Secondly, the sensors yield data on the tissue responses to toxins, in particular regarding the integrity of the cellular membrane and the intracellular cell death process. They also provide important information regarding specific cellular functions and their recovery capability after a toxic insult. The final choice of the microsensors aimed to monitor cellular functions will be made in function of the list of reference compounds.

Results

Engineering of cells for non-invasive, imaging-based assessment of cell toxicity and death: We have inserted Flippase Recognition Target sites into AAVS1 (Adeno-associated virus integration site 1) using optimised ZFN nucleases in hPSC. The cassette that was introduced in the AAVS1 site consists of a PGK promoter encoding a hygromycin resistance cassette (HygroR) and a thymidine kinase cassette (HSV-TK), flanked by Flippase Recognition Target sites. Upon excision of the sequence between the FRT sites and replacement with the sensor cassette, gancyclovir-based negative selection of cells will allow isolation of correctly recombined clones (a process named recombinase mediated cassette exchange, RMCE). We have demonstrated that introduction of and CAAGS promoter driving GFP can be achieved by RMCE (Figure 4.10).

Figure 4.10 RMCE of biosensors in AAVS1 locus.
We have created and integrated NF-κB, p53 and caspase-3 reporter cassettes into AAVS1 (Adeno-associated virus integration site 1) using Flippase recombinase, tested the activity of the different cassettes and demonstrated (Figure 4.10) that all three cassettes function in cell line models. They will now be introduced by RMCE in the AAVS1 locus of iPSCs. Once demonstrated that the recombination has occurred correctly, it will be of importance to establish that the activation of NF-κB, caspase-3 and p53 reporters in the engineered iPS cells is similar to unmodified cells. Subsequently, cells will be suitable for long-term toxicity testing.

**Development of sensors for real-time basic culture conditions:** Here we wish to detect soluble molecules as chemical indicators of cellular damage as well as overall health of the culture. We distinguish different levels of information and corresponding methodology: this task serves to develop sensor modules directed towards medium composition, e.g. pH, oxygen, glucose etc., that will allow continuous monitoring of the culture conditions, which could then, in a second phase, lead to continuous adjustment of the media composition to enhance cell differentiation and health. Such electronic sensors are, in part, readily available commercially. In Table 4.1, we summarise the sensors under development.

**Table 4.1 Status of microsensor developments.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>Too much noise in the signal; setup optimisation ongoing</td>
</tr>
<tr>
<td>Lactate</td>
<td>Achieved</td>
</tr>
<tr>
<td>pH</td>
<td>Ongoing</td>
</tr>
<tr>
<td>Oxygen</td>
<td>Initial setup difficult to integrate</td>
</tr>
<tr>
<td></td>
<td>Integration of O₂ dye in microchannel walls</td>
</tr>
<tr>
<td>K⁺</td>
<td>Only cell necrosis</td>
</tr>
<tr>
<td></td>
<td>Sensor response not stable over two weeks</td>
</tr>
<tr>
<td></td>
<td>Optimisation of design and fabrication process</td>
</tr>
<tr>
<td>Alanine transaminase</td>
<td>Ongoing</td>
</tr>
<tr>
<td>NH₄⁺ / Urea</td>
<td>Sensor response not stable over two weeks</td>
</tr>
<tr>
<td></td>
<td>Optimisation of design and fabrication process</td>
</tr>
</tbody>
</table>

Aside from the creation of the different sensors, we also aim to incorporate these in the microfluidic channels. During the first year of **HeMiBio**, we decided that it would be most appropriate that sensor blocks are built that:
Allow construction of systems with gradually increasing complexity (start with modules without integrated sensors and add sensors later on).

Allow design and fabrication of user specific systems with combinations of various fluidic and sensor modules.

Figure 4.11 Incorporation of microsensors.

4.3.3 Generation of Sequentially More Complex Bioreactors

State of the Art

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

In this context, microfabrication and microfluidics are the key factors for success, as they allow for the dynamic control of the cellular microenvironment at the microscale (Anderson & van den Berg, 2004). Cells and cellular complexes cultured in a microfluidic device can be addressed by a variety of soluble and mechanical factors. The technology allows for the study
of a cellular response to stimuli that cannot be created in a static culture. Flat-plate bioreactors have been used to study hepatocyte function and differentiation. Using this model, it was shown that the metabolic function of hepatocytes is significantly reduced when the cells are exposed to shear >5 dyne/cm². While reducing shear flow reduces mechanical damage, it also reduces oxygen and nutrient delivery. Strategies for protecting cells from shear include seeding the cells in groves (Park et al., 2005) or microwells (Khademhosseini et al., 2005). The flat-plate bioreactor has been used to culture hepatocytes under a stable oxygen and hormone gradient in vitro. Cultured hepatocytes show aspects of zonal differentiation into the oxygen-rich and –poor regions respectively, consistent with in vivo zonation (Allen & Bhatia, 2003).

The packed-bed reactor is a variant in which hepatocyte aggregates are perfused in an environment that allows for 3D-organisation (Powers et al., 2002; Strain & Neuberger, 2002). The integration of the heterotypic cell-cell interactions is an additional level of complexity required for capturing the function of the in vivo liver. We hypothesise that the packed-bed type reactor holds the greatest promise due to its ability to support long-term function of 3D-tissue aggregates, its low shear (resembling that in the intact liver sinusoids; Lalor et al., 2002), and minimal well-to-well variance.

**Approach**

We are generating sequentially more complex bioreactors to culture hepatocytes, stellate and endothelial cells for >28 days. We hypothesise that this will lead to further maturation of immature cells derived from iPS cells and assure their persistent differentiated and quiescent state for lengthy periods of time. We will test whether (immature) hepatocytes, LSEC and HSC can be captured from mixed iPS cells cultures by microfluidic isolation on hepatocyte, LSEC and HSC-specific antibody-micropatterned surfaces. This will also allow testing if such micro-patterned co-cultures support differentiation and long-term maintenance of liver-specific functions. Differentiated cells will then be released and allowed to self-assemble into organoids in 3D multi-well bioreactors. The function of the different electronic microsensors and the molecular sensors introduced in the cells will be tested first in the 2D-bioreactors and, if validated, subsequently incorporated in the 3D-bioreactors. We will also ensure that the molecular and electronic sensors provide the information aimed for, i.e. the continuous (intermittent) in vivo assessment of the differentiated state of the three cellular components, their overall state of health and ultimate function and survival of the liver-like tissue in vitro.

As the ultimate goal of HeMiBio is to use the to-be-created device as an alternative to rodent toxicology studies, it will be of the utmost importance to evaluate if the 3D-bioreactors reveal the toxicity expected from a number of prototypical hepatotoxic compounds known to trigger clear-cut liver injuries in vivo. As proof-of-concept, the 3D-bioartificial liver-device will be treated with test compounds with suspected toxicity. A prerequisite to accomplish this critical
task is the establishment of a set of function and toxicity screening assays, as well as a list of reference compounds, which is currently work in progress by the compound selection Working Group (see chapter 4.10.2).

Results

Development of 2D bioreactor for isolation-patterning: We fabricated polydimethylsiloxane (PDMS) stencils by replica moulding of PDMS on SU8 defined silicon masters. Stencils contained through holes ranging in diameter from 100 to 900 μm. This allowed us to differentially pattern hepatocytes and endothelial cells, as shown in Figure 4.12. Such micropatterned cultures have been previously shown to maintain the function of primary rat and human hepatocytes for over 41 days in vitro, a period of time deemed sufficient for second-dose toxicity testing. The next generation of devices will be used to both capture and pattern iPS cell-derived cells. Using these patterns, we aim to study the effect of heterotypic cell-cell interactions on iPS cell-derived hepatocyte metabolism.

Figure 4.12 Microfluidic micropatterned-reactor design and selective capture of hepatocyte and endothelial cell lines.
The second bioreactor for which a prototype was developed is a flat-plate stainless steel bioreactor on top of micropatterned glass. Cells in this bioreactor are allowed to aggregate in microfabricated wells, creating a 3D organoid we had previously shown to maintain its function up to 50 days in vitro. A hepatocyte-endothelial micro-aggregate is shown in Figure 4.13.

![Figure 4.13 Flat bioreactor.](image)

**Fabricated at Hebrew university an Fraunhofer IZM**

**Designed to be same size as petridish with 0.5 ml contents**

**Next Steps**

We propose to test our devices using Medicyte Upcyte® hepatocytes co-cultured with Upcyte® MVEC, LSEC or HSC. Subsequently purified iPS cells-derived hepatocytes, LSEC and HSC will be tested. The latter will allow testing if fluorescence can be used to test whether genetically modified iPS cells-derived cells respond to stresses, apoptosis or genome instability (NF-κB, caspase, and p53, see above).

The final design of the bioreactor will be a 3D packed bed bioreactor (graphically represented in Figure 4.14, that has 4 critical components: (1) a high-throughput microfluidics addressable array, (2) a plastic and glass housing, (3) a sensor integrated multi-well plate, and (4) a filter matrix on which the cells sit. Bioreactor dimensions have been designed to support the seeding of hepatocytes, and equal numbers of non-parenchymal cells under physiological shear and normoxic conditions. Cells are expected to pack 250-500μm high, the approximate length of a hepatic sinusoid, possibly giving rise to metabolic zonation.
Two different prototypes have been designed, using rapid multi-component assembly (IMEC) and hot embossing (FRAUNHOFER) also shown in Figure 4.14. The first prototype (IMEC) allows rapid changes in the basic design, which will allow us to optimise the bioreactor, while the second prototype (FRAUNHOFER) allows for rapid industrial-scale fabrication, which is our end goal. Their suitability for cell culture as well as the ease of design and fabrication are now being evaluated. In addition, we are commencing the integration of the sensor unit depicted in Figure 4.11.

4.3.4 Innovation

Sensors: In this project, we propose to integrate further microsensors with 3D-liver bioreactors. Commercial sensors, aimed to monitor cell culture conditions, and microfabricated microsensors intended to detect specific hepatic functions in each microwell, will be integrated in a microfluidic cartridge. Here, we plan to generate microsensors that are able to have a stable signal for up to four weeks. Ion-selective sensors such as potassium-selective microelectrodes able to monitor and quantify cell death in real-time, as well as ammonium-selective microelectrodes intended to detect the detoxification capability of a hepatocyte population, will be fabricated. Similarly, enzymatic microsensors, such as alanine transaminase and CYP450, will be created to monitor early signs of dysfunction after exposure to toxic compounds.
**Micropatterned, flat-bed and 3D bioreactors:** By integrating micropatterning with a flat plate bioreactor, we will introduce a one-step isolation-patterning of mixed cultures of differentiated iPS cells. Such micropatterned co-cultures of primary hepatocytes and fibroblasts were shown to maintain *in vivo* levels of liver-specific gene expression and function following 41 days in culture, recapturing drug-toxicity events in static cultures. Novel to our approach is that we use a self-renewing source of cells that can generate all cellular components required for creation of a liver-simulating device using a common differentiation method. However, the frequency of differentiated cells in iPS cells cultures is low, making cell purification by traditional techniques difficult, with few cells surviving at the end of the process. The laminar flow in microfluidic devices coupled with antibody-coated surfaces allows for the efficient capture of minute cell populations with minimal damage. This combination of micropatterning and microfluidic isolation offers a simple, yet elegant, technique to generate organ-simulating environment under flow.

Our design adapts the proven LiverChip design to support multicellular aggregates, introduces an innovative microfluidic gradient generator enabling the rapid generation of experimental matrices for high-throughput screening, and, more importantly, integrates a novel set of biological and chemo-electrical sensors that enable the continuous measurement of cellular function and health.

**4.3.5 Cross-Cluster Cooperation**

Regarding the selection of model compounds to be tested in the bioreactor, there has been a continuous interaction between *HeMiBio*, DETECTIVE and ToxBank. Specifically, the *HeMiBio* partner “Vrije Universiteit Brussel” has proposed a set of chemicals on behalf of *HeMiBio*. Being a partner of the DETECTIVE project as well, this partner also proposed this list of compounds to the DETECTIVE consortium. The proposal was sent to ToxBank, which is responsible for the selection of chemicals for the entire SEURAT-1 project cluster. Teleconferences dealing with compound selection, organized by ToxBank, were consistently attended by them. A final list of chemicals was recently established.

We worked extensively together with ToxBank in relation to the design of the data warehouse. This included hands-on demonstration of the type of studies to be done by many partners in *HeMiBio*, and description of data sets generated from these experiments. In addition, we beta tested the initial formats of the data upload software sets.

We plan in the summer of 2012 discussions with SCR&Tox and NOTOX, consortia that are also using established bioreactors, to determine how to streamline work done in *HeMiBio* with aim of generating improved bioreactors suitable for testing long term toxicity and experiments being done by the other two consortia. During these meetings we will also discuss the types of cells to be tested in parallel in the existing reactors and the advanced reactors described...
in chapter 4.3.2. In addition, discussions will be held with NOTOX related to ‘-omics’ aspects of hepatocytes, under optimal culture conditions, and exposed to chronic toxins, using the list generated by the SEURAT-1 Research Initiative.

4.3.6 Expected Progress within the Second Year

We will further elaborate on zinc-finger-mediated homologous recombination and insert additional marker cassettes into the different cell types (hepatocytes, stellate cells, endothelial cells). Cell culture conditions will be optimised for the expansion of quiescent iPSC-derived cells. Ion-selective microelectrodes will be optimised and equipped with a pre-concentration stage, and the 3D prototype bioreactors will be made available by the end of the second year.

4.3.7 Future Perspectives

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

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

A second area where the technologies developed in HeMiBio could be used to create an organ-simulating device is the kidney. 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 3 main components. Blood flows into the nephron by 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. There, 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 a highly concentrated waste urine. Several methods have been developed to isolate glomeruli and culture the three types of glomerular cells. For instance, the concept of a nephron-on-a-chip using a MEMS-based (MicroElectroMechanical System) bioartificial device has been proposed, but attempts to populate this device with the various renal cell types that constitute a kidney have not been reported (Weinberg et al., 2008). However, these methods suffer from impure cell populations and the short life span 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 up to one month, forming a 3D glomerular organoid (Wang & Takezawa, 2005). The population of 2D- and 3D-bioreactors with hiPS cell-derived cultures, enabling life imaging and monitoring of the differentiated cell types as is presented by HeMiBio, could also be used to develop bioartificial renal technology.

Although the liver is the principal organ to clear toxins from the body and, therefore, is the most vulnerable target for the latter, certain drugs may be toxic to other vital organs, such as the heart, the blood vessels or the brain. In order to predict toxicity of cosmetic compounds or drugs to these organ tissues, creation of devices that mimic their architecture and function for toxicity screening is also of great importance. As for the liver, the functional, morphological and molecular characteristics of the cells that constitute these organs are determined by the environmental factors (e.g. the vicinity to and direct contact with other cell types in the organ, exposure to flow and certain oxygen levels, etc.). All these parameters can be integrated in a bioreactor system, like the one we propose here for the liver. The technology developed in the HeMiBio project, i.e. cells that are manipulated as such that their differentiation state, functionality and viability can be monitored, including the 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 a need in animal testing.
References


**Recent key references of project members (last 3 years) related to the field of research**


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

*Jürgen Hescheler*¹

4.4.1 Introduction and Objectives

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

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

This report highlights the first functional and ‘-omics’ experiments carried out in the first year. Upon evaluation of the most appropriate human cellular model system for each organ, respectively, and after comprehensive assessment of the relevance of an initial list of specific compounds, first experiments have been undertaken. Such experiments served the purpose of reviewing the cellular model systems and the assessment of corresponding functional readouts. The data generated identified a preliminary list of up- or down-regulated genes on a transcriptomic level and moreover, elucidated the sub-lethal compound concentrations that may be suitable to subsequently investigate repeated dose toxicity. Based on these initial results first exposure protocols have been established mimicking the long-term application of chemicals.

¹ - On behalf of the DETECTIVE consortium
In addition, sample preparation protocols have been put in place to accommodate work in the field of '-omics' readouts, which will be the focus of the second year of the project.

The overall aim of DETECTIVE is to identify, develop and evaluate relevant *in vitro* biomarkers and surrogate endpoints that can be used for safety assessments of chronically acting toxicants relevant for humans. Objectives in the first project year are listed below.

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

- In collaboration with SCR&Tox, evaluating the suitability and robustness of existing cell lines for use in developing biomarkers for repeated dose toxicity testing *in vitro*.

- Developing functional readouts in human *in vitro* model systems mainly for liver, heart and kidney and, possibly, also for other model systems as provided by other building blocks. These functional parameters include i) electrical activity (*ECG*-like, MEA), ii) impedance measurements, iii) imaging, and iv) cell-specific functional readouts such as enzyme activities, cytokine release, albumin and urea secretion, glycogen uptake, cholestasis, steatosis, and protein release from target cells.

- Developing '-omics' readouts in human *in vitro* model systems for liver, heart and kidney and possibly also for other model systems as provided by other projects of the SEURAT-1 Research Initiative. These '-omics' readouts include: i) integrative transcriptomics (microarrays for global screening of gene expression, epigenetics, and miRNA), ii) proteomics, and iii) metabonomics.

- Developing concepts for a standardised approach employing appropriate cellular model systems that allow i) identifying the best candidates for toxicity assessments with regard to reproducibility (biomarker qualification) and ii) distinguishing sensitive and target-specific biomarkers from generic cellular stress effects.

- Systematically organising data with the use of standardised nomenclature that facilitates the online sharing of biomarker metadata.

The work carried out to achieve the third and fourth objective listed above is highlighted in this report.

The acceptance and use of biomarkers for regulatory purposes is a major task that has yet to be accomplished. Indeed, it requires a set of quality evaluations to determine the scientific
validity of the proposed biomarkers, such as information on the predictivity of the biomarker itself, but also the methodologies by which it can be assessed.

### 4.4.2 Cellular Systems and Compound Selection

Cellular systems in DETECTIVE represent three target organs, namely liver, heart, and kidney. During the first year of the project, the DETECTIVE partners carried out the first functional and ‘-omics’ experiments with positive and negative controls. The goal was to:

- Identify one suitable cellular model system for each target organ to be used in the experiments, as, due to their labour intensity and high costs, ‘-omics’ techniques can only be applied to one test system for each target organ;
- Validate the usability of cellular systems with regards to functional readouts;
- Validate the applicability of cellular systems to long-term and repeated dose toxicity testing;
- Identify first effective marker genes and
- Define exposure protocols for repeated dose toxicity testing.

It was initially agreed to test a small number of positive control compounds for the evaluation of the applicability of a given cellular system for repeated dose toxicity testing. These compounds were applied in order to compare the different cell systems available. One positive control and one negative control compound have been subjected to testing in the available cellular systems. Subsequently, first functional and ‘-omics’ data has been generated to accommodate selection of one cell system per organ group that were suitable with respects to functional readouts, demonstrating sufficient reproducibility and robustness. The selected *in vitro* systems will then be employed for further ‘-omics’ and functional analyses.

For the ‘liver group’, in order to identify one single cell system to be used for further repeated dose toxicity testing, gene expression profiling was carried out on 5 different systems treated with a positive control (paracetamol). This also allowed identifying the relevant concentrations and defining a first exposure protocol for repeated dose toxicity.

For the ‘heart group’, the first step was to evaluate the usability of one human cellular system suitable for the various functional readouts. As some of the readouts systems were based on working with murine cells, they had to be adapted to human systems. Also, any human cellular system had to be reviewed with regards to their characteristics and suitability for the various readouts. For example, hESC-derived cardiomyocytes currently lack sufficient purities (>97% required), which renders them insufficient for impedance measurements. The data generated
so far allowed defining first exposure protocols for repeated dose toxicity testing.

A second step in the ‘heart group’ was to define exposure protocols suitable for repeated dose toxicity testing. This was done by the assessment of all initial data and, based on concentrations identified to represent sub-lethal levels, exposure protocols were put into place for different time points. It is further planned that microarray experiments should be performed in parallel with real time monitoring electrophysiological properties and cytotoxicity in the presence of compounds by employing the xCELLigence technology. This will allow establishing whether changes of the gene expression pattern in the presence of toxicants correlate with the functional characteristics. Similar protocols have been in place within the ‘kidney group’ and will be adopted for the ‘liver group’ as well.

For the ‘kidney group’, who had already established a particular cellular system, RPTEC/TERT1, the first step was to test different compounds (12) applying impedance measurements (xCELLigence system) to evaluate the suitability of the readout. It also allowed defining first exposure protocols for repeated dose toxicity.

In order to identify the first putative marker genes, transcriptomic analyses were carried out, involving one positive and one negative control, compound specific for each respective organ. These very preliminary results will have to be confirmed by more extensive experiments, applying more compounds in appropriate exposure protocols. To accommodate more extensive ‘-omics’ experiments, which will be in focus for the second year of the project, sample preparation protocols have been established.

As for the relevant chemicals to be tested, each ‘organ group’ proposed compounds suggested or known to be relevant for their respective cellular systems. Subsequently, in close cooperation with ToxBank, all available data on such compounds has been compiled and reviewed to assist in the selection of relevant compounds to be tested as positive and negative controls.

In a more long-term approach for DETECTIVE, the project partner ‘Fraunhofer Institute of Toxicology and Experimental Medicine’ is currently evaluating their data bank of more than 1000 compounds analysed in 28-days and 90-days studies to identify the most common mechanisms leading to the problems in liver, kidney and heart. Corresponding to these mechanisms, an initial set of at least 4 positive and 4 negative control compounds may be selected to be tested in the in vitro systems for liver, heart and kidney toxicity, resulting in ‘-omics’ and functional data generation. Upon successful completion, a further (training) set of 10 positive and 10 negative compounds may be used for analyses through ‘-omics’ and several functional readouts, as well as imaging assays to identify a list of biomarkers or assays which discriminate well. Finally, a further 15 positive and 15 negative compounds (“test set”) may be analysed only with the list of best biomarkers/assays identified before and thus, sensitivity and specificity will be determined.
4.4.3 Functional Readouts

State of the Art

High-content image analyses, as well as impedance measurements provide the unique possibility for continuous monitoring of major cellular aspects such as migration (Harrill et al., 2010), proliferation (Ohta et al., 2012), cell morphology, cell-cell interactions, and colony formation. These functional measurements are, thus, very adequate for repeated dose experiments (Malin et al., 2011). Further development and adaptation of these technologies to long-term toxicity tests will allow screening of large amounts of substances for non-specific as well as for target organ-specific effects. Since screening tests are designed to be highly sensitive, sometimes at the expense of the specificity of the test, we will assess the predictive value of used screening tests. Continuous readout systems will be accompanied by established technologies such as measurement of electric activity using MEA or cell type specific readouts (Liang et al., 2010).

Detailed information about the technologies used for the functional readouts and how they can lead to the development of novel toxicity biomarkers are given in the first volume of this Annual Report (Hescheler, 2011).

Approach

Electrical activity: The electrophysiological characteristics of cardiomyocytes upon repeated dose exposures have been monitored in real-time using multielectrode arrays. QT interval is the most important functional parameter to measure cardiotoxicity. MEA and xCELLigence technologies are used to monitor the effect of doxorubicin and isoproterenol, at different concentrations in single and repeated dose scenarios, on beating iPS cells from murine and human sources.

Impedance measurements: The possibility of using the xCELLigence system for repeated dose exposures on renal epithelial monolayers and cardiomyocytes was explored in the first project year. The DETECTIVE partners ‘Medizinische Universität Innsbruck’ and ‘Roche’ collaborated to investigate the applicability of this system to nephrotoxicity studies. Until now there has been little experience with transporting epithelial cells on the xCELLigence system. RPTECT/TERT1 cells (Evercyte, Vienna) (Wieser et al., 2008) were seeded at different densities on xCELLigence E-Plates and monitored over 16 days; the cell index was measured at regular intervals. In a second round of experiments, the cells were treated with specific nephrotoxins.

In the case of the cardiomyocytes, recordings of cellular impedance were performed with
xCELLigence Cardio instrument (Roche Diagnostics) on a 96 well format. Before each experiment and following cell addition, background impedance signals were recorded every 10 min for each of 20 milliseconds. Raw data are displayed by the measurement as cell-index values, which are calculated from the changes of the impedance signals. Data was analysed using Real-Time-Cell-Analyser (RTCA) software (version1).

**High throughput imaging:** The objective for the first year regarding cardiac toxicity measurements was to develop an automated movie analysis conducted by partner ‘Joint Research Centre’ using a conventional microscope equipped with a high-speed digital camera that allows the detection of effects on the contractile activity of cardiomyocytes at low costs.

With reference to hepatocytes, partner ‘Universiteit Leiden’ has worked on: i) the development of assays to analyse stress and cytotoxic effects, ii) automated high resolution imaging of toxic effects and iii) the analysis of toxicological effects using time lapse microscopy. The reporter cell lines for these studies are obtained by BAC transgenomics (Hyman et al, 2008). A cassette containing the fluorescent moiety (GFP) and selection markers is introduced at the N- and/or C- terminus of the corresponding gene in a Bacterial Artificial Chromosome (BAC). These BAC-GFPs are transfected into mammalian cells and selected for stable integration in the genome; our current model cell line are HepG2 cells. A BAC construct contains the endogenous gene promoter and all the surrounding intron sequences, including (unknown) regulation mechanisms to maintain the endogenous transcription and protein translation levels, making it a highly suitable *in vitro* toxicity assay model for imaging-based readouts.

**Cell type specific functional readouts:** The primary objective was to quantify well-established cell type specific functions with respect to their necessity for correct toxicity evaluation, since some of these assays are more time-consuming and less applicable for high throughput screening compared to the reporter assays and assays established in the other functional readouts. In cooperation with these partners, it is being investigated which of the more laborious conventional assays can be replaced by less time-consuming innovative assays without losing predictive power.

**Results**

**Electrical activity:** The results of the MEA experiment with doxorubicin are summarised in Figure 4.15. With higher doses, significant decrease of beating frequency was observed, suggesting a possible toxic manifestation. The dose-dependent cardio toxic effects from these experiments have helped designing an appropriate exposure protocol for human based iPS-derived cardiomyocytes.
Figure 4.15 MEA experiment. (A) Representative phase-contrast image of human iPSC-derived beating cluster (day 16) plated on MEA chamber. (B) Screenshots of representative raw tracings to illustrate the dose-dependent morphological change of beating frequency by doxorubicin. (C) Effect of doxorubicin on beating frequency of hiPS cell cluster (© UKK, unpublished data)

Impedance Measurement: The results of the experiments using renal epithelial monolayers demonstrate the usefulness of the system for monitoring both the viable cell number and the barrier function. It can be observed that as cells form domes (an indicator of vectorial transport of water and solutes), CI decreases to an oscillating plateau (Figure 4.16). Moreover, the exposure to several nephrotoxins shows distinct patterns of response. The system holds great promise for long-term repeat dose experiments of renal epithelial cells, as it is highly sensitive, has high temporal resolution, is non-invasive and fully automated.

Figure 4.16 Image of Dome formation on xCELLigence E-Plates recorded on a Cellavista Imaging System and xCELLigence profiles of RPTEC/TERT1 on xCELLigence E-Plates (© Roche Diagnostics)
Consequently, the xCELLigence system proved to provide extremely sensitive and highly temporal alteration on renal epithelial function (i.e. increased cell index corresponding to decreased barrier function), toxicity (decreased cell index corresponding to loss of cells from the monolayer due to cell death), cardiac cell index measure and dose determination for short and long term toxicity testing.

The DETECTIVE partner ‘University of Cologne’ has validated the quality of hiPS-derived cardiomyocytes and studied dose response resulting from doxorubicin exposure over a period of 14 days using the xCELLigence system to detect the sub-lethal dose that could be used for long-term repeated dose studies (Figure 4.17).

The cell index exponentially increases for the first 50 hours of cell seeding and reaches a plateau stage until 336 hours (14 days), thereafter cells start detaching and eventually the cell-index drops significantly by 720 hours (30 day; Figure 4.17A). This data suggests a testing window for pharmacological substances for in vitro toxicity testing platform. An estimated time of 14 days can be used as a period of repeated dose testing strategy that can avoid noise-signal ratio of the test system. Beating rates, frequencies and amplitudes are shown in Figure 4.17D, E, F, and G, respectively. The data is presented for every 24 hours after plating of cells on E-plates. Note that initially (6 hours) cells do not show the characterised beating pattern and are in acclimatisation phase. By 12 hours, beating frequency is normalised, but with a higher beating rate. Beating rate and frequency is regular on and after 48 hours up to 384 hours (15 days) and is in fidelity limits (20% ±, shown as bracket in panel F). Thereafter, an irregular beating is observed, which also suggests a definitive testing period for cardio toxicity testing platform.
Figure 4.17 Characterisation of hiPS cell derived cardiomyocytes. A) The cell attachment measured as a factor of cell index (y-axis) for a period of 720 hours using Roche xCELLigence system. B) Phase-contrast cell pictures taken on 48 hours, 336 hours and 720 hours. Cells are observed to slog off the surface during later time points. Scale bar corresponds to 100µ. C) Cardiac specific markers tested using real-time PCR. D, E, F, G) Data showing the beating rate/frequencies/amplitude measured using xCELLigence. The numerical data presented are mean ± standard deviation (© UKK, unpublished data).
Furthermore, a dose selection study for repeated dose toxicity testing was conducted (Figure 4.18). Doxorubicin at various concentrations was introduced after 48 hours of seeding and the cell index as a measurement of cell attachment/proliferation/death was recorded over time. Higher concentration of the drug (2.5, 5 and 10μM) are observed to be cytotoxic and instantly kill cells within hours of introduction (<12 hours). Middle range concentration (0.625 and 1.25 μM) shows delayed cytotoxicity (>40 hours). However, at lower concentrations (<0.3μM and lower) cells resist and do not show significant cell death (Figure 4.18A). For a long-term repeated dose study, such concentrations should be suitable. The beating rate is seen to be significantly increased by a dose of 0.3 and 0.156μM in a time-dependent manner (Figure 4.18B). It is highest after 48 hours of treatment at this concentration, which is also the half-life time of the drug in a human patient.

**Figure 4.18 Dose selection for repeated dose study. A) The cell index measurement for cardiomyocytes on xCELLigence. The scoring was performed for up to 100 hours post cell seeding. B) Beating rate measurements (© UKK, unpublished data).**
High throughput imaging: In the most basic approach of cardiac toxicity measurements, microscopy imaging techniques can be applied to test substances for their capacity to interfere with the frequency of contractions, thus resulting in the so-called chronotropic effects (Figure 4.19). A standard operation procedure was established detailing the steps for an efficient use of an automated or semi-automated movie analysis of contracting cardiomyocytes and its application for the measurement of beating frequency.

**Figure 4.19** Microscope setting (© JRC technical report “Development of a novel endpoint for the validation of embryonic stem cells test.”)

Such system has been developed using murine stem cell-derived contracting cardiomyocytes (according to INVITTOX protocol #113). First sets of recordings have been performed with a positive chronotrope (isoprenaline, Figure 4.20), henceforth to be adopted for human cellular systems.

**Figure 4.20** Chart depicting pixel brightness changes in time before and after treatment with a positive chronotrope (isoprenaline) (© JRC, unpublished data).

The analysis of cardiomyocyte beating is usually conducted by microscope inspection of cultured cardiomyocytes. A programme for analysis of high-speed movies of beating cardiomyo-
cytes has been developed and validated. It allows qualification and quantification of beating frequency in cardiomyocytes.

In the hepatotoxicity studies performed by partner ‘Universiteit Leiden’, a library of 10 stable transgenic cell lines has been produced. *Table 4.2* summarises the planned and already successful GFP-reporter cell lines. For each construct, multiple clones have been obtained during the selection procedure, which were subsequently monitored by high-resolution confocal microscopy to validate the BAC-GFP clones as accurate markers for the organelle and stress response type they stand to model. Only those clones that show accurate distribution of the reporter were selected as representative reporter cell lines. For CYC1 it is GFP in the mitochondria, with a homogenous distribution (*Figure 4.21*).

*Table 4.2* Current status of reporter cell lines

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Reporter for</th>
<th>GFP construct</th>
<th>Stable Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Srxn1</td>
<td>Oxidative stress</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>NFE2L2</td>
<td>Oxidative stress</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>TP53</td>
<td>DNA damage</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>BRCA1</td>
<td>DNA damage</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>TP53BP1</td>
<td>DNA damage</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>PRPF40A</td>
<td>Nuclear morphology</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>ACTB</td>
<td>Morphology</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>ACTN1</td>
<td>Morphology</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>MYH9</td>
<td>Morphology</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>VIM</td>
<td>Morphology</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>CDH1</td>
<td>Morphology</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>VCL</td>
<td>Morphology</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>HSPA5</td>
<td>ER stress</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>PDI6</td>
<td>ER stress</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>XBP1</td>
<td>ER stress</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>LC3</td>
<td>Autophagy</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>KEAP1</td>
<td>Ox. Stress / Autophagy</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>TMM23</td>
<td>Mitochondria</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CYC1</td>
<td>Mitochondria</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CYCS</td>
<td>Mitochondria</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>ATF4</td>
<td>ER stress</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>DDIT3</td>
<td>ER stress</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>Nanog</td>
<td>Pluripotency</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>NFKBIA</td>
<td>Inflammation</td>
<td>X</td>
<td>-</td>
</tr>
<tr>
<td>RELA</td>
<td>Inflammation</td>
<td>X</td>
<td>-</td>
</tr>
</tbody>
</table>

*Figure 4.21* HepG2 CYC1-GFP reporter single clone cell lines. Each picture represents a different isolated CYC1-GFP clone.
Afterwards, the successful clones were subjected to the compounds with well-described intracellular cell stress mode of actions, such as tunicamycin for endoplasmatic reticulum (ER) stress, \textit{m}-chlorophenyl hydrazone for mitochondrial membrane depolarisation and cytochalasin D to disrupt the actin network. By laser scanning confocal microscopy, the GFP reporter distribution between control and treatment were compared. For most reporters, results of this test delivered the expected phenotype- summarised in Table 4.3.

<table>
<thead>
<tr>
<th>Reporter for</th>
<th>Tagged Gene</th>
<th>Tested Compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeletal Integrity</td>
<td>ACTB</td>
<td>nocodazole (+), cytochalasin D (+), Y27632 (+)</td>
</tr>
<tr>
<td>Mitochondria Morphology</td>
<td>CYC1, TMM23</td>
<td>\textit{m}-chlorophenyl hydrazone (+), oligomycin A (+), carbamazepine (-), diclofenac (-), antimycin A (+)</td>
</tr>
<tr>
<td>Oxidative Stress</td>
<td>SRXN1, KEAP1</td>
<td>acetaminophen (+), diclofenac (+)</td>
</tr>
<tr>
<td>UPR / ER Stress</td>
<td>HSPA5, XBP1, PDIA6</td>
<td>tunicamycin (+), thapsigargin (+), brefeldin A (+), carbamazepine (+), diclofenac (+)</td>
</tr>
<tr>
<td>DNA damage</td>
<td>PRPF40A</td>
<td>doxorubicin (-), cisplatin (+)</td>
</tr>
<tr>
<td>Autophagy</td>
<td>LC3</td>
<td>All of above the tested clone did not respond</td>
</tr>
</tbody>
</table>

Taking into account the forthcoming repeated dose toxicity studies, the effect of acetaminophen treatment was explored in HepG2 cells expressing the Nrf2 response gene Srxn1 coupled to GFP. This reporter cell line is sensitive to induction of oxidative stress. Using the automated imaging and quantification techniques, it was possible to show that acetaminophen induces Nrf2 responses at doses above 1mM (Figure 4.22).

**Fig 4.22** Live cell imaging of HepG2 Srxn1-GFP cells shows concentration-dependent responses to consortium compound acetaminophen. (© UL, unpublished data)
In summary, BAC transgenomics has been used for the first time to produce an *in vitro* model system for hepato-cellular toxicity. Compound exposures show dose-dependent effects at the protein level of several specific reporter lines. The preliminary results show that using this setup will allow performing high throughput high resolution based imaging to phenotype stress responses induced by model compounds.

**Cell type specific functional readouts**: 20 different standard operating procedures of cell type specific functional assays have been compiled during the first year. Selected test substances with known toxicity will be applied to compare the predictive power of these well-established functional tests for novel biomarkers identification.

### 4.4.4 ‘-omics’ readouts

**State of the Art**

In contrast to *in vitro* assays for toxicology providing a dose-response relationship for a particular combination of test substance and cellular system that are unable to assess the dynamic aspects of the cellular response to a toxin, analyses of toxicogenomics datasets indicate that compounds affect a variety of cell biological processes. So far, these different processes cannot be determined by a unified toxic endpoint and, thus, a battery of complementary functional and ‘-omics’ readouts needs to be employed.

To this end, it may be of major relevance to study e.g. epigenomic alterations as a mechanism underlying repeated dose (chronic) toxicity. It is of particular relevance to test the hypothesis that epigenomic changes induced by model compounds for repeated dose toxicity persist in the *in vitro* models upon ending exposure. There is a growing body of evidence that chemical exposure can induce changes in the DNA methylation patterns, indicating that a full understanding of this type of epigenetic change is required to gain insight into the molecular mechanisms of action (Rusiecki et al., 2008). As such, DNA methylation analysis may contribute to biomarker identification for the prediction of chemical toxicity, particularly in combination with gene expression analysis and other markers of epigenetic changes. Post-translational modification of histones, together with DNA methylation, is often referred to as the epigenome. Histone modifications may affect DNA methylation, which has been correlated with deacetylation of histones 3 and 4, along with shifts in histone methylation patterns (Fuks, 2005). It is the dynamic nature of these histone modifications that renders them particularly susceptible to environmental influences and, thus, stabilisation of histone acetylation patterns or alteration by external stimuli, can be major responses to chemical exposures and may be involved in gene expression mediated toxic responses.

Further readouts will be employed complementing transcriptomic and epigenomic analyses.
MicroRNAs (miRNAs), have emerged as powerful negative regulators of mRNA levels in several systems (Hudder & Novak, 2008), influencing mRNA levels of important genes involved in metabolic and toxicological pathways. Moreover, miRNAs have been implicated in biological processes such as normal development and disease pathology, particularly in cancer (Farazi et al., 2011).

Proteomics are a mean to assess early/immediate biological responses which are not detectable by transcriptomic technologies. These posttranslational modifications have a direct impact on enzyme activities and protein-protein interactions. Gene transcription is only activated at the downstream stages. Usually the first response after stimulation of cells can be seen in phosphorylation of heat shock proteins and other components of stress responses (Groebbe et al., 2010).

The systematic analysis of metabolism (metabolomics) in living organisms, alongside genomics, epigenomics, transcriptomics and proteomics, is increasingly being viewed as a vital part of the toolkit for global biomolecular modelling (systems biology) and biomarker discovery. Metabolic profiling as a means of biomarker discovery and metabolic biomarkers themselves have several potential advantages over genomic and proteomic counterparts: metabolites are a defined chemical entity irrespective of species, genotype, localisation and biological matrix, facilitating the translation of analytical procedures between models and man; changes in metabolism are a phenotypic and often functional endpoint (Keun & Athersuch, 2007).

**Approach**

**Gene expression profiling:** In the first year, initial gene expression studies were carried out. For instance, the ‘liver group’ identified the most appropriate cellular systems for further elucidation of biomarkers by comparing Affymetrix gene array data of controls with cells incubated with in vivo relevant concentrations of paracetamol. The assessment of sub-lethal concentrations in a given cellular system was first established by a viability (MTT) assay. Subsequently, the effect of exposure of cells to paracetamol in IC10 concentrations was investigated by microarray analysis.

**Other ‘-omics’ readouts:** Some preparatory work has been carried out for epigenetic, miRNA, proteomic and metabolomic analysis for experiments to start in the second project period.

**Results**

**Gene expression profiling:** The IC10 (10% inhibitory concentration) was determined based on MTT experiments (in triplicates) and exemplary results are given in Figure 4.23.
The exemplary experiments shown here evaluated the applicability of hepatocyte-like cells derived from human progenitor cells as an *in vitro* cellular model for the identification of biomarkers related to hepatic toxicity, exhibiting relatively high IC10 values.

Furthermore, a comparison of gene array data of liver cellular systems was performed by principle component analysis (*Figure 4.24*). It included primary human hepatocytes (freshly isolated cells from three donors), hepatocyte like cells (HLCs) from progenitor cells, and HLCs from human embryonic stem cells. To be able to compare the aforementioned cell types to immature cells, several independent batches (biological replicates) of human embryonic stem cells were additionally included. The results demonstrate a relatively large distance between the primary human hepatocytes and the other cell types. *Figure 4.24* also differentiates between the “HLC islets” and mixed HLC populations from human embryonic stem cells. The “HLC islets” represent cells with a hepatocyte like morphology, expressing albumin and the polarity marker DPP IV (*Figure 4.25*), as evidenced by immunostaining. They were micro-dissected from the hESC mixed cell population to specifically analyse the “hepatocyte like cells”. However, even this cell population seemed very distant from primary human hepatocytes if genome-wide RNA expression patterns were considered.

*Figure 4.23* Example of IC<sub>10</sub> Determination (© Data processed by MasterPlex ReaderFit software)

*Figure 4.24* Principal component analysis of different cell types (© IFADO, VUB unpublished data)
Figure 4.25 Comparative co-staining in hESC derived Hepatocyte Like Cells (HLC) and primary hepatocytes with albumin and bile canalicular marker DPPIV after 18 days of differentiation. HLCs express low levels of albumin and the polarity marker DPPIV. (© IFADO unpublished data).

Similarly, HepG2 and HepaRG cells showed huge differences compared to primary human hepatocytes (Figure 4.26). However, it should be considered that the Affymetrix gene array data for HepaRG cells were obtained from a publicly available database. The ‘liver group’ yet has to test their own cells to guarantee that the differentiation process was performed according to quality standards (work in progress).
When human hepatocytes were incubated with acetaminophen, systematic differences were already observed at 200 mM (Figure 4.27). This concentration corresponds to maximal plasma concentrations at therapeutic doses. Even higher differences were obtained at 1000 and 5000 mM corresponding to blood concentrations that are associated with an increased risk of hepatotoxicity in vivo.

**Figure 4.26** Principal component analysis of different cell types (© IFADO, VUB unpublished data, Jennen et al. 2010 - EBI database).

**Figure 4.27** Primary human hepatocytes: 24 hours APAP incubation with 200, 1000 and 500 µM (© NIBIO- Genomics assisted Toxicity evaluation system).
It was agreed that the systematic biomarker identification within the ‘liver group’ will be performed with primary human hepatocytes. The other cell types express a number of hepatocyte markers and represent an important achievement in stem cell research. Nevertheless, considering genome-wide RNA patterns, the differences to primary human hepatocytes are too large, leading to an excessively high risk of irrelevant biomarkers being identified. A remarkable result is that the human hepatocyte in vitro system shows up- and down-regulated biomarkers already after incubation with concentrations corresponding to human plasma concentrations of acetaminophen at therapeutic doses.

4.4.5 Innovation

With regard to the research highlighted in this report, DETECTIVE has the following innovative characteristics:

- Cellular models used for the readouts include primary cells, human cell lines, hES cell-derived cells and, importantly, iPS cell-derived cells as a promising alternative to hES cells. The partner ‘University of Cologne’ has successfully generated iPS cell-derived cardiomyocytes, which are used in DETECTIVE. As iPS cells have only been described recently and have not yet been studied extensively, hES cells are included for reference purposes as the commonly recognised reference system for evaluating iPS cells.

- With limited scope of availability of analytical tools for MEA, the partner ‘University of Cologne’ is working on developing such a software tool, which would analyse electrogram data in the context of in vitro toxicology.

- Novel imaging technologies as well as impedance measurements now provide the unique possibility for continuous observation of major cellular aspects, such as migration, proliferation, cell morphology, cell-cell interactions and colony formation. These functional measurements are, thus, highly suitable for repeated dose experiments.

- For innovative biomarker development, DETECTIVE will now integrate multiple data streams derived from transcriptomics, miRNA analysis, epigenetics, proteomics and metabolomics with traditional toxicological and histopathological endpoint evaluation, in view of a systems biology or, rather, systems toxicology approach (in cooperation with the other building blocks of the SEURAT-1 Research Initiative).

- For developing novel intermediate biomarkers in combination with in vitro endpoints for repeated dose toxicity, such integrations are specific research goals. The first results available from evaluation of the cellular systems are a proof of principle. For example, work on primary human liver cells has shown
remarkable results indicating cell response after incubation with concentrations corresponding to human plasma concentrations at therapeutic doses.

In this pathway-based approach, DETECTIVE will thus identify which ‘toxicological signatures’ have a high predictive value. It will systematically assemble highly sensitive and specific, standardised toxicity biomarkers derived from relevant human cellular sources.

4.4.6 Cross-cluster Cooperation

The DETECTIVE consortium ensures strong collaboration with the other projects of the SEURAT-1 Research Initiative, aiming to avoid duplication of effort and ensuring optimal synergy between projects.

The DETECTIVE consortium has sought cluster level cooperation from SCR&Tox, which has successfully established and maintained discrete cellular phenotypes in stable cultures of interest to be tested by partners in DETECTIVE, with regards to functional and ‘-omics’ readouts. Also, DETECTIVE has approached SCR&Tox for improving existing protocols for differentiation of human embryonic stem cells towards the cardiac lineage. Discussions were held regarding the sharing of cellular systems for evaluation and selection of the most ideal cell source to be used by the consortium.

During the process of compound selection, DETECTIVE has approached ToxBank for their expert advice on compounds, assessing specific toxicity to the various organ tissues – heart, liver and kidney. Extensive exchange of knowledge regarding a whole range of interesting compounds has eventually resulted in lists of compounds recommended to be most relevant and useful to be tested by the respective ‘target organ groups’. Elaborate discussions have also taken place between various DETECTIVE partners and ToxBank on statistical data analysis and data management of compound specific dose response data. Field visits were organised for the representatives from ToxBank to determine the nature of data analysis support tools and services required. The DETECTIVE data analysis partners have also taken part in virtual meetings organised by ToxBank on the SEURAT-1 ‘Gold Compound Working Group’ (GCWG) and the ‘Data Analysis Working Group’ (DAWG).

A cluster level cooperation with NOTOX is currently ongoing to discuss certain characteristic features of primary human liver cells. NOTOX is known to have conducted extensive long term in silico toxicity prediction studies using this particular cell system.

4.4.7 Expected Progress within the Second Year

During the second year, DETECTIVE will focus on more extensive ‘-omics’ analyses, as well as developing suitable treatment protocols elucidating the identification of biomarkers of chronic toxicity:
Continuation of repeated dose toxicity testing for additional compounds using functional readouts and gene expression analysis
Launch of the first epigenetic and proteomic experiments
Refinement of exposure protocols for repeated dose toxicity testing
Set-up of the database for analysis and storage of experimental results

**DETECTIVE** will aim at developing a method for the integration of MEA-based measurements with the Roche xCELLigence system. In order to achieve this, the contraction of the cardiomyocytes is recorded using the cell sensor impedance technology and the same parameters will be deduced from the contraction profile. This allows a comparison with the electrographic recording and a determination whether the electric potentials correlate with the magnitudes of the contraction. Differences between both profiles may allow additional insights into the functional coupling of both events in treated cardiomyocytes. The development of software tools for the analysis of MEA readings for safety testing will be further completed. Based on MEA analysis expertise, custom-made user-friendly software for analysing MEA readings will be developed for analysing electrogram data in the context of *in vitro* toxicology using MATLAB or a similar framework.

The assessment of the short- and long-term (repeated dose) hepatotoxic effects of compounds using the cell sensor impedance technology will be finalised. Cells differentiated from the induced pluripotent stem cells will be seeded at different densities in xCELLigence plates. Adhesion on different extracellular matrix proteins and long-term survival using different culture media are optimised and recorded for the provided cell lines. Dose response curves of short- and long-term (repeated dose) toxicity with different compounds (up to 7 concentrations) with known toxic effects on hepatocytes are recorded. Different compounds will be categorised in heat maps according to profiles. This is used for compound toxicity profiling and for selecting optimum time points for expression and imaging analysis. It is also the basis for the detection of new toxicity biomarkers. Reversibility of the compound effect will be investigated by washing out the compound and monitoring the effect.

As for the renal system, it has been demonstrated that the xCELLigence system provides extremely sensitive and highly temporal alteration on renal epithelial function (i.e. increased CI corresponding to decreased barrier function) and toxicity (decreased CI corresponding to loss of cells from the monolayer due to cell death). The xCELLigence system will, thus, be further exploited towards the development of repeated dose testing regimes for more extensive ‘-omics’ analysis.

The effect on the cells will also be recorded with imaging methods analysing for different toxicity readouts and general changes in cell morphology. The second year of the **DETECTIVE** project also comprises the final phase of the development process of assays related to analyse stress, cytoskeletal rearrangements, apoptotic and necrotic effects in cardiomyocytes.
and hepatocytes on the Cellavista imager. Specific labelling reagents allowing the imaging of apoptotic events such as annexin V binding, caspase activation and others will be evaluated on cardiomyocytes and hepatocytes and tested using model compounds with known toxic effects. Other reagents for labelling disruption of the cell membrane, the cytoskeletal architecture, or indicative of oxidative stress are tested in the same way. Software for automated calculation of the affected cells is developed in parallel and is used for the analysis.

A robust and consistent gene expression methodology for toxicological approaches using hESC will be delivered. For this, RNA from various differentiation points will be isolated in order to determine the time point of differentiation in which no further differentiation occurs. It can then be assumed that only entirely differentiated somatic cells are available at this point. This time point will be the optimal one to perform repeated dose experiments. In the first two years, given the huge dynamic range and chemical diversity of proteins, optimisation, separation and differential quantification, are the major first line analytical challenges related to proteomics. This will be addressed by the end of second year by evaluating different SOPs for LC-MS and PAGE analysis, pooling and fractionation strategies and by cross-validation with other building blocks and methods like integration of proteomic signatures with findings from transcriptomics and metabolomics. Furthermore, by the end of second year, metabolomic analysis of the first sets of cell samples will be completed. To achieve this, focus will be given to the continuing development of SOPs for GC-MS and LC-MS analysis and normalisation techniques. NMR based techniques have already been developed that are capable of detecting extracellular metabolic changes related to chemical treatment in in vitro cell systems.

Regarding epigenetics analyses, basic questions have been clarified, including the development of SOPs for the different partners for isolation of samples, SOPs for subsequent ‘-omics’ analyses, technical procedures, and data analyses tools. A test study using the liver model of choice has been designed in order to generate a reference database as positive control for the ‘-omics’ readouts. Subsequent epigenetic, transcriptomic, miRNA and proteomic analyses and experiments will be the focus of the second project year. The lists of compounds proposed by ToxBank analysing data on DNA methylation, histone modification, and miRNA, as well as further screening of literature for models used (in vitro or in vivo), dose, exposure time, effects on epigenomic readouts, and mode of action will lead to sound rationales with regards to the application of relevant refined exposure protocols for long-term toxicity to determine the biologically effective doses relevant for alternative testing, the next important short-term goal into year 2 of the project.

In parallel, the plan for storing the experimental data by the DETECTIVE project, which has been developed in the first year, will be carried out in the second year by implementing and setting up all the planned databases and tools. It involves both installing existing database solutions, such as BII database for ‘-omics’ data, as well as developing new resources such as the database for functional data.
Finally, a first version of selected endpoints suitable for industry and a compendium of verified, stable and easy-to-measure functional and '-omics' biomarkers will be drafted, including GLP-compliant SOPs for selected, most relevant biomarkers.

In a more medium-term perspective, upon establishment of stable cell systems and organ-simulating devices provided by SCR&Tox and HeMiBio, a battery of complementary functional and '-omics' technologies will be fully applied to generate comprehensive data for the selected compounds and exposure protocols. Larger test sets of positive and negative control compounds will be analysed with the most relevant biomarkers/assays identified and their sensitivity and specificity will be validated. Towards the end of the project, functional readouts will be employed to relate the results of the '-omics' readouts to the physiological status of the cells to aid qualification of the '-omics' markers. Stabilisation, selection of final biomarkers and verification of those in another laboratory or using a second method will be the emphasis in the last project year.

Future long-term perspectives in this research area comprise the development of defined, validated and routinely applied workflows, including the electrical activity and impedance measurements, high throughput imaging, gene expression profiling, epigenetics, proteomics and metabolomics to establish a solid and reliable basis on which a future in vitro test system used by the industry can be built on. The integration of these functional as well as '-omics' systems in a work process shall give rise to a comprehensive and modular screening platform for specific approaches in cellular analysis, such as the cytotoxicity and stem cell differentiation. DETECTIVE results will enable the introduction of novel testing strategies, leading to significant productivity gains, which in the future will make it possible to evaluate more candidate drugs or cosmetics substances, thereby creating opportunities for more speculative and creative approaches to drug research and to the planning of a possible SEURAT-2 Research Initiative.

### 4.4.8 Future Perspectives

Successful completion of DETECTIVE will change our understanding of repeated dose toxicity testing methods, subsequently leading to a screening pipeline of functional and '-omics' technologies, including high content and high throughput screening platforms, to develop and investigate human biomarkers for repeated dose toxicity in human cellular in vitro models.

Establishment, selection and verification of highly predictive biomarkers in a pathway- and evidence-based approach constitutes a major building block in an integrated approach towards the replacement of animal testing in human safety assessment and will lay the foundation for subsequent efforts in SEURAT-2. The following Research Initiative could address the limited scope of DETECTIVE / SEURAT-1, which mainly covers the use of limited number of human primary cellular systems and test compounds. The employment of several more, in future available human ES/iPS cell-derived systems and the testing of a more extensive range of
toxicological substances is just one effort to further broaden our knowledge on long-term toxicity. This expansion in data and the resulting knowledge will be most relevant in establishing a solid and reliable basis on which future *in vitro* test systems used by the industry can be built upon. The scientific expertise related to detection of endpoints and biomarkers for repeated dose toxicity, derived by the end of the DETECTIVE project, will lay the foundation to a proof-of-concept-based detailed road map to a novel repeated dose toxicity *in vitro* testing platform. Such should be one aspect of a SEURAT-2 Research Initiative - testing and assessing several other human cell systems and establishing high throughput screening platforms for various drug libraries.
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4.5 COSMOS: Integrated *in Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety

**COSMOS**

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4.5.1 Introduction and Objectives

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

Currently, these models are simplistic and do not fully capture the repeated dose effects of cosmetics to humans. This is partially a result of insufficient data due to historical and poorly maintained databases as well as the complexity of the endpoint to be modelled. The current knowledge gaps are illustrated and summarised in Figure 4.28.
Figure 4.28 Summary of the knowledge gaps preventing the assessment of the safety of cosmetic ingredients to humans from computational techniques.

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

The specific objectives of COSMOS are:

- To collate and curate new sources of toxicological data and information from regulatory submissions and the literature.
- To create an inventory of known cosmetic ingredients and populate with chemical structures.
To establish thresholds of toxicological concern for endpoints relating to human repeated dose toxicity.

To develop innovative strategies based around categories, grouping and read-across to predict toxicity and relate to adverse outcome pathways where possible.

To establish kinetic and PBPK models, in vitro, in silico and other relevant data to predict target organ concentrations and long term toxicity to humans.

To integrate open source and open access modelling approaches into adaptable and flexible in silico workflows using the KNIME technology.

In the following, five aspects of the work towards these objectives and the results achieved so far to solve the underlying challenges are highlighted and discussed.

4.5.2 Building the COSMOS Database Framework

Background

The management and sharing of chemical, biological and toxicity data in a flexible and sustainable manner play a central role within the COSMOS project. The TTC concept and (Q)SARs are two important approaches to predict the toxicological effects of chemical compounds in human beings. The availability of data in these domains affects the performance and applicability of the results of predictive toxicology projects. Data sets likely to be of use to the COSMOS project, suitable for the development of in silico models, have been identified. These data sources are mainly divided into three toxicological endpoints, repeated dose toxicity, mutagenicity / carcinogenicity and reproductive toxicity.

In order to facilitate the development of new models and predictive approaches, an effective collection and governance strategy of toxicological data sets is required. The data are collected, curated, quality-controlled, stored and managed in a flexible and sustainable manner to support the predictive modelling tasks within COSMOS and the SEURAT-1 Research Strategy. It is expected that the data volume will increase while usage of specific sub-sets of data for modelling workflows with various purposes will also require additional functionalities, and drive the future usage of the COSMOS repository. Data usage is also expected to increase and refine in specific modelling applications, and such foreseen challenges are taken into account at the current analysis and design stages of the COSMOS database.

The research undertaken covers three main directions.

- An overview of toxicity data sources and studies.
- The design of the data scheme and the data entry tool of the prototype database.
- The definition of a data curation strategy.
These represent fundamental components of the COSMOS state-of-the-art database and web-based retrieval system, which will serve for sharing resources, models and supporting workflow developments for Cosmetics Europe, the personal care products industry, policy makers in the European Commission and Member State authorities, and the predictive toxicology community in general. Moreover, data are being collected from various sources and, as a result, the challenges of quality definition and management have to be addressed, as well as of standard workflow methodologies having user actions and data reliability as core data management functionalities.

State of the art

There is a well-recognised paucity of toxicological data for most mammalian endpoints and repeated dose toxicity in particular (Cronin, 2009). A further particular concern is whether the available animal data have any relevance to human exposure. Some databases containing repeated dose toxicity or carcinogenicity data are publicly available. However, the exact relevance of these databases for cosmetics has yet to be established. This area is further complicated by the lack of open databases and current confusion over ontology for toxicological endpoints in the field of repeated dose systemic toxicity testing. There is, however, an opportunity to harvest existing data relating to cosmetics and colorants, both from within the industry and public sources such as regulatory agencies.

The European Commission CosIng database provides a list of over 20,000 cosmetic ingredients with links to SCCS (Scientific Committee on Consumer Safety) opinions. There are other lists of chemicals associated with cosmetics, e.g. from the United States Food and Drug Administration (US FDA) and the Personal Care Product Council (PCPC). However, there is no single inventory of cosmetic ingredients. Further, no inventory is currently available which contains high quality and validated chemical structures associated with it. Such an inventory is required to assist with the understanding of chemical space, and to enable chemical grouping.

Approach

Current advances in data storage and collaborative processing in predictive toxicology require flexible representation of data as resulting from (quality) data curation. Predictive toxicology requires multidisciplinary data and generates numerous, diverse and multiple-source chemical, biological and toxicological resources. Improved and consistent use of such challenging toxicity data requires a new integrated vision in this domain: data governance in predictive toxicology to provide a new framework to integrate quality check and flexible data sharing functionalities is proposed. Besides the quality of data, data governance also considers the authorisation of data and the availability of data as shown in Figure 4.29.
There is therefore a clear rationale and significant demand for a data governance system for the COSMOS repository. This should permit easy input, storage and access of data for organisations using the COSMOS database prototype. In addition it will provide a secure data repository that can be accessed by relevant stakeholders within and outside of the SEURAT-1 Research Initiative. This will allow for efficient benchmarking, better reporting of model development for risk assessment and also for expeditious policy and decision making.

Results

The data model (for the capture of toxicological information) designed within COSMOS exhibits appropriate complexities. It reflects the diverse and heterogeneous layout of toxicological, chemical and biological data resources. In principle, the data model currently comprises two major areas which are interconnected – the chemical and the toxicological area (see Figure 4.30), together with the collaborative framework for data sharing functionalities. These are the foundations for a further update to a data governance framework.
The chemical area (or part) of the data model is ‘compound-centred’, i.e., all entities stored in this area are related to the entity ‘Compound’, which represents a chemical compound or substance. In this model, a compound (or substance) is a chemical composition, which may consist of one or more molecules (‘Structure’ entities). Irrespective of the presence of real chemical structure(s) in a ‘Compound’ instance, any related information items can be stored. There are numerous information items stored in the data model, e.g., registry numbers, different identifiers employed, physicochemical properties of compounds, literature references, etc. The set of information items has been identified from the present database content.

The toxicological part of the model reflects recent developments of ToxML (Yang et al., 2008). Therefore, most of the toxicological information stored in the ToxML format could be successfully parsed and stored in the COSMOS database without losing data granularity or relations.

At the very high level, the toxicological part of the data model defines the entity ‘Study’ which reflects a toxicological study. Each ‘Study’ instance may in its turn consist of a various number of ‘Test’ instances. In fact, a test represents a series of experiments applied to a ‘Test System’ which might be a series of animals, tissues, etc. The ‘Test System’ instances reflect all the peculiarities of tests carried out. Each test references a ‘Test Result’. The ‘Test Result’ reports the genetic toxicity or carcinogenicity outcomes of the relevant toxicity experimental series. Finally, a ‘Study’ relates to its ‘Study Result’ which aggregates the results of separate tests carried out in frames of the study. For example, the ‘Study Result’ is a final outcome of either the genetic toxicity or carcinogenicity endpoint based on all test outcomes within a study. Other ‘Study’ and ‘Test Results’ include LOEL and NOEL values.

The direct access to the COSMOS database, i.e., by directly executing SQL statements to read, modify or remove data, is quite difficult as the numerous entities have multiple dependencies. Thus, an end-user who would need to append a new compound to the database would have to
ensure that all these dependencies are taken into account and create many database objects manually. Accordingly, the COSMOSDB web application is designed to serve the purpose of data access. Currently, it provides search and reporting capabilities only, with ensured extensibility for future versions for, e.g., data entry functionality.

As access to the data in the COSMOS database should occur over the network and multiple users should be able to perform data operations in parallel, the client-server architecture of the system has been chosen and realised as it serves such an operational model at best. The three-tier architecture of the web application allows for several levels of abstraction between the end-users and real data in the database. These layers ensure the extensibility of the functional spectrum of the application, providing also enough performance bandwidth to each single user.

Currently, the COSMOSDB system is set-up on the high-performance computer facilities of COSMOS partner ‘University of Bradford’ who hosts the production version of the system. A dedicated server (the COSMOS sun server), for hardware and software specifications will ensure that future versions of the COSMOSDB system can handle multiple user requests and searches in parallel. The server employs a virtualisation technology (containers and zones) that allows for a secure, flexible, scalable and light-weighted operation and administration of the system, including authentication issues, maintenance, backup and further developments (Oracle, 2011). The COSMOS server consists of different zones which can be regarded as individual machine with different levels of exposure to the network (global and non-global, either Intra- or Internet). The COSMOS server has been configured with one ‘global’ (cosmos) and five ‘non-global’ zones (cosmosdev, cosmsolive, cosmosweb, cosmosdb and cosmoliux).

4.5.3 The COSMOS TTC Datasets

Background

In development of alternatives to animal testing, the TTC approach can serve as one of the practical safety assessment tools for chemicals for which no in vivo testing results are available. It is a risk assessment paradigm that establishes a human exposure threshold value for chemicals, below which there is a low probability of an appreciable risk to human health. This approach is an extension of the Threshold of Regulation (TOR) adopted by the US Food and Drug Administration for substances used in food-contact articles (FDA, 1995). The original TTC concept used a single threshold for all chemicals, based on the conservative assumption that an untested chemical could pose a cancer hazard. It was subsequently expanded to include non-cancer endpoints by Munro et al. (1996).

One goal of the COSMOS project is to extend the current TTC approach to cosmetic ingredients. The current non-cancer TTC assessment is based on the dataset used by Munro et al. (1996), which contains 613 diverse tested chemicals and their NOEL (No Effect Level)
values from oral repeat dose toxicity studies. Transforming the data to chronic NOAELs (No Adverse Effect Levels), Munro et al. (1996) identified the 5th percentile of the cumulative distribution for each Cramer Class (Cramer et al., 1978) and devised the current thresholds. COSMOS addresses the key issues in applying the current approach to cosmetic ingredients, including: i) the applicability of the chemical domain of the non-cancer database (Blackburn et al., 2005); ii) the applicability of the Cramer Decision Tree for protectiveness (Blackburn et al., 2005); and iii) extrapolation of the oral-to-dermal route exposures (Kroes et al., 2007).

For these three issues, the following approaches have been adopted: i) the chemical domain of the current Munro database may need to be expanded to sufficiently cover cosmetic ingredients; ii) the Cramer Decision Trees may be modified to reflect biological pathways; iii) the target organ dose extrapolation due to oral-to-dermal exposure differences may be overcome by incorporating ADME (absorption, distribution, metabolism, and excretion) knowledge. To this end, COSMOS has also 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 cosmetic ingredients and Expert Group 2 for the evaluation of oral-to-dermal extrapolation. In collaboration with the Expert Groups, a non-cancer TTC dataset for cosmetic ingredients (COSMOS TTC v1.0) has been compiled (Worth et al., 2012). Furthermore, data collection activities for regulatory NOAELs, skin penetration, oral absorptivity, and skin/liver metabolic differences have been initiated. PBPK modelling is also used for the target organ dose validation. In the following, the COSMOS TTC v1.0 dataset and the preliminary analysis will be highlighted.

State of the Art

Some years ago, Blackburn et al. (2005) compared the above-mentioned dataset compiled by Munro et al. (1996) (613 substances) with 248 chemicals used in personal and household care products (PHCP) from the repeat dose toxicity database at Procter & Gamble. The intention was to confirm that the chemical categories represented in the original analysis conducted by Munro et al. (1996) were broad enough to include the categories of compounds selected from ingredients known to be used in consumer products. The authors further evaluated whether the chemicals used in PHCP from each Cramer Class (Cramer et al., 1978) (but not included in the analysis carried out by Munro et al.) would have NOELs that would fall within the range of the NOELs identified in the original analysis by Munro et al.

This was an important milestone since it provides the guidance for structural categories and analysis approaches to compare between different datasets. More recent analyses confirm that in a broad sense the dataset from Munro et al. (1996) is chemically diverse (Bassan et al., 2011; Worth et al., 2012); however, it should also be noted that the 248 chemicals used in PHCP from Procter & Gamble may not adequately cover the chemical domain of the much larger inventories of cosmetic ingredients. For example, this analysis conducted in
2005 categorised chemicals in Munro (613) and PHCP (248) datasets based on 92 structural categories; 66 out of 92 categories were adopted from the Cosmetic Toiletries and Fragrance Association. Eleven categories populated in chemicals used in PHCP were missing in the Munro dataset. These categories were: alkoxylated amines, alkoxylated carboxylic acids, alkyl sulfates, benzophenones, betaines, fatty acids, fatty alcohols, glyceryl esters and derivatives, polymeric ethers, and sarcosinates/sarcosines. In nine additional categories, the Munro dataset has much lower representation (30% lower in population) as compared with the PHCP dataset. These additional categories include well-known cosmetic ingredients: alkyl aryl sulfonates, alkylamido alkylamines, amino acids, carbohydrates, colour additives, long chain, siloxanes and silanes, soaps, and sorbitan derivatives. In addition, 37 categories were only present in the Munro dataset, which amounts to over 50% of the structural categories missing in either Munro or PHCP. Furthermore, the study provides further cautions concerning the limited availability of toxicity data; for example, only 45 tested chemicals within the Cosmetic Toiletries and Fragrance Association categories were included in the analysis. Therefore, to evaluate the applicability of the TTC approach to cosmetic ingredients, it warrants a compilation of a new non-cancer TTC dataset that is enriched with cosmetic ingredients and more suitable toxicity data.

Currently, most of the reliable data are experiments from the oral route of exposure. Oral repeated dose toxicity data are available from a few structured database sources including US FDA and US EPA, Fraunhofer ITEM RepDose and the OECD QSAR Toolbox. Regulatory NOEL/NOAEL values are also available from the European Commission Scientific Committee on Consumer Safety (EC SCCS), the European Chemicals Agency (ECHA), and the US EPA Integrated Risk Information System (IRIS) as monograms/opinions.

Approach

The COSMOS approach to evaluate the applicability of the TTC approach to cosmetic ingredients was to first establish a large comprehensive inventory of cosmetic ingredients (i.e., the Cosmetics Inventory). Cheminformatics techniques have been applied to join the two sources, the European Commission’s CosIng database (EC CosIng database, 2012) and the list from the US Personal Care Products Council (2012). Cheminformatics software techniques have also been employed to classify compounds into structural categories for reproducible and efficient classifications. Using the Cosmetics Inventory as the reference library, a dataset providing NOEL values from repeated dose oral toxicity studies has been established as a seed for the new non-cancer TTC dataset of cosmetic ingredients (COSMOS TTC dataset). The NOEL values are obtained from reliable regulatory databases such as US FDA and EPA. Data from the EC SCCS and ECHA are being added. The biggest challenge is to compile reliable toxicity data and to develop a set of decision rules to determine NOAEL values from various sources, which in turn can be merged and updated with the Munro dataset.
Results
The Cosmetics Inventory v1.0, established based on the above-mentioned sources, provides 9,883 unique CAS registration numbers (RNs) and 20,598 unique INCI (International Nomenclature of Cosmetic Ingredients) names and is used as a reference library of cosmetic ingredients. The Venn Diagram in Figure 4.31 illustrates the overlap between the CosIng and PCPC inventories by CAS registration numbers as well as INCI names. They clearly indicate that there are many-to-many relationships between them. The overlap was used to define the COSMOS Cosmetics Inventory v1.0, a set of 4460 unique chemical structures that are found in CosIng and/or PCPC, with connection tables, to be used for chemical domain and TTC analysis.

Figure 4.31 Sources of chemicals in the COSMOS Cosmetics Inventory.

To establish a new non-cancer TTC dataset for cosmetics, two different aspects of the data domain are required. One is the chemical database that defines the cosmetic ingredients and the other is the oral repeat dose toxicity database that provides dose-response level information so that NOEL/LOEL can be confirmed or determined. Based on the available databases from Munro, US FDA (PAFA and OFAS), US EPA (ToxRefDB) and US NTP (National Toxicological Program) a superset of oral repeat dose toxicity data was compiled. Of the 2,000 tested chemicals within this superset, 660 unique cosmetic ingredients were found in the Cosmetics Inventory v1.0. From this collection, a set of 558 unique chemical structures containing NOEL/NOAEL values was used to assess the chemical domain and for further TTC analysis.

The COSMOS datasets were characterised by employing structure and subgraph features, as well as physicochemical property descriptors (see chapter 4.5.4).

To assess the degree of protectiveness provided by the Cramer-related threshold values for cosmetic ingredients, the thresholds derived from the Munro (Munro et al., 1996) and Cosmetics TTC v1.0 datasets were compared using a cumulative distribution analysis of
NOEL values. As mentioned above, the COSMOS TTC dataset was derived from multiple data sources (Munro, FDA PAFA, EPA ToxRefDB, EPA IRIS, SCCS, ECHA) and is subject to on-going extension and revision within COSMOS. For simplicity and conservativeness, the distribution analysis was applied to the lowest NOEL value for each substance in the dataset, which may not be the NOAEL. 385 structurally well-defined substances were considered, excluding inorganics, organometallics, polymers, and substances for which the tested form was unknown. This analysis included developmental and reproductive toxicity studies, but excluded all repeated dose studies with exposure duration shorter than a subchronic study (typically 90 days). The NOEL values from subchronic studies were divided by a factor of 3 (Munro adjustment factor for subchronic to chronic conversion) (EC CosIng database). The NOEL values for each Cramer Class are compared in Figure 4.32 for the Munro and the COSMOS TTC v1.0 datasets (Jacobs et al., 2012).

Figure 4.32 Cumulative distribution functions of the Munro and COSMOS TTC v1.0 datasets. (Red=Cramer Class I, Blue=Cramer Class II, Green=Cramer Class III).

Table 4.4 summarises the 5th percentile NOEL for the substances in each Cramer class. Although the three cumulative distribution function curves (Figure 4.32) were clearly non-normal, a theoretical log-normal distribution was assumed for this calculation rather than using a non-parametric approach, since the use of data from the full distribution gives more robust estimates of the percentiles.

Table 4.4 Distribution analysis of TTC datasets: COSMOS TTC v1.0 and Munro.

<table>
<thead>
<tr>
<th>Cramer Class</th>
<th>Human Exposure Threshold (μg person⁻¹ day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>COSMOS TTC v1.0</td>
</tr>
<tr>
<td>Class I</td>
<td>817</td>
</tr>
<tr>
<td>Class II</td>
<td>866</td>
</tr>
<tr>
<td>Class III</td>
<td>170</td>
</tr>
</tbody>
</table>
It is worth noting that the Cramer Class I of the cosmetic ingredients has a lower 5th percentile NOEL than the Munro TTC dataset. For example, the 5th percentile derived from the COSMOS TTC dataset (1362 μg/kg/day) is lower than the corresponding Munro value (3000 μg/kg/day) by a factor of ~2. Nevertheless, our preliminary analysis, based on the use of the Cramer scheme (Cramer et al., 1978) and the prior identification of potential genotoxicants, concludes that the current TTC approach is broadly applicable to cosmetics. However, a number of improvements could be made, through the quality control of the underlying TTC datasets, modest revisions/extensions of the Cramer classification tree, and development of explicit guidance on how to apply the TTC approach. This analysis has been published as a JRC report (Worth et al., 2012).

4.5.4 Characterisation of the Cosmetics Chemical Space

Background

A characterisation of the chemical space of the cosmetics inventory was performed in order to optimise in silico methods, such as (Q)SAR, grouping and read-across, for the purpose of long-term toxicity prediction of cosmetic ingredients. The main objective was to assess the chemical similarities and dissimilarities (and identifying relations between its constituents, if possible) in the two COSMOS datasets. The COSMOS non-cancer TTC dataset (version 1.0), containing repeated dose toxicity data for cosmetic ingredients, and the COSMOS Cosmetics Inventory (version 1.0), a compilation of substances from the EU CosIng and US PCPC lists, were used for this purpose.

This preliminary analysis explored the applicability of the TTC approach to cosmetic ingredients. Thus the chemical space of the two COSMOS datasets was compared with the chemical space of the Munro non-cancer dataset, which is de facto the TTC dataset for non-cancer endpoints, to assess whether this underlying TTC dataset is representative of the ‘world’ of cosmetic ingredients, as represented by the COSMOS Cosmetics Inventory and the COSMOS TTC dataset.

State of the Art

The chemical space is a representation of the structural features and/or molecular properties covered by a defined set of chemicals. The molecular properties may include intrinsic properties that are defined purely by chemical structure (e.g. size and shape), or derivative properties (e.g. chemical reactivity), as well as extrinsic and biologically relevant properties such as metabolic behaviour. Chemoinformatic methods allow for the visualisation and characterisation of the chemical space in a consistent manner, so that different datasets (including regulatory inventories and datasets suitable for model development) can be compared. Such comparisons enable the identification of regions of overlap and divergence, as a basis for targeted model development, testing, and/or regulatory action.
The general challenge of any chemical (or molecular) similarity analysis is related to the structure representation, mathematically encoded in ‘structural descriptors’. In addition, the definition of similarity is not trivial and depends on the particular aims of the investigation. It has to be emphasised that a present-day concept of similarity and diversity should not focus on the molecular scaffold alone, but also include the physicochemical property space to facilitate mechanistic interpretation of future models. Therefore the combined use of structural subgraph features and holistic descriptors also representing the physicochemical properties of the molecules is recommended in order to better characterise the inventory of interest. The more information about the system under investigation exists, the better choice on the types of descriptors for an appropriate structure representation can be made.

Approach

A structural similarity analysis usually comprises two main steps. Firstly, the chemical structures to be compared are represented in terms of descriptors which encode their constitutional, topological, geometrical and/or surface and physicochemical properties. A second step involves a quantitative comparison of those descriptors using similarity (or dissimilarity) analysis methods.

Thus, the statistical or intellectual selection of an initial set of descriptors for the structure representation of the COSMOS datasets was the first and crucial step of the characterisation of their chemical space. A careful evaluation of several types of structure representations was performed and a small number of descriptors was selected, including physicochemical properties, structure and subgraph features together with use categories.

Results

The COSMOS datasets were characterised by employing physical/chemical property descriptors. The same descriptors were employed to characterise the Munro non-cancer dataset (see previous chapter 4.5.3).

The structure features were identified either by defining the SMARTS (a language for describing molecular patterns) representations for substructure searching using RDK in KNIME (see chapter 4.5.6) or subgraph features in the MOSES fingerprinter (Molecular Networks GmbH). The subgraph features, developed by the US FDA Center for Food Safety and Applied Nutrition, are grouped by types of atom, bond, ring, functions and connectivity. They were coded in the Chemical Subgraph Representation Mark-up Language (CSRML) format1, which can be used to represent features that cannot be easily written in SMARTS. The analysis based on structure and subgraph features was used to characterise and compare the datasets in terms of structural classes. Various structural categories of Munro, Cosmetics Inventory v1.0, and COSMOS TTC v1.0 are compared in Figure 4.33.

1 - http://bulletin.acscinf.org/node/224#W7
Although the COSMOS TTC v1.0 dataset lacks in steroids, the dataset populates all other classes defined by the CTFA (Cosmetic Toiletries and Fragrance Association) that Munro et al. (1996) lacked. The COSMOS TTC v1.0 dataset, in particular, enriches the classes of long aliphatic chains, glycol ethers, ketones, and non-ionic alcohol ethoxylate surfactants. A detailed analysis of the chemical domains of these datasets has been carried out, including the differentiation of the datasets by physicochemical properties based on the structural differences. Furthermore, the COSMOS TTC v1.0 dataset is also representative of all the substance use types found in the COSMOS Cosmetics Inventory v1.0. The most highly populated use types include skin-care (conditioning/moisturisers), emulsifiers, perfumes (fragrances), hair dyes, colorants, and UV absorbers/filters, antimicrobials, vitamins, and plasticisers.

*Figure 4.33 Structural classes in the Cosmetics Inventory (red), in the COSMOS TTC dataset (green), in the Munro dataset (blue).*

Furthermore, the chemical space of the COSMOS Cosmetics Inventory was also characterised and compared with the Munro et al. (1996) and the COSMOS TTC dataset by means of easily interpretable physicochemical properties representing size (molecular weight), shape (diameter, number of rotatable bonds), partitioning behaviour (log P), solubility (log S), general characteristics of the structures (H acceptors, H donors) and reactivity (dipole, HOMO, LUMO energies, electronegativity, hardness, softness and electrophilicity). These physicochemical properties were calculated by using the ADRIANA. Code software (Molecular Networks GmbH, version 2.2.4) and MOPAC (MOPAC2009, JJP Stewart, Stewart Computational Chemistry, Colorado Springs, CO, USA 2009) for the reactivity descriptors.
The median values of the most informative descriptors of the COSMOS Cosmetics Inventory, the Munro and the COSMOS TTC datasets were compared by means of a radar chart (Figure 4.34). The analysis of the radar chart showed that (i) the Munro dataset and the COSMOS Cosmetics Inventory contain larger structures (higher molecular weight) than the COSMOS TTC dataset. In more detail the analysis showed that despite of these differences more than 85% of the structures have molecular weight less or equal than 400 g/mol in the three datasets; (ii) the COSMOS Cosmetics Inventory has a higher number of structures with long linear chains (higher number of rotatable bonds and diameter); (iii) the COSMOS TTC dataset has a higher prevalence of hydrophilic chemicals (lower log P values).

![Radar chart of the most informative molecular descriptors of the COSMOS Cosmetics Inventory (median values; Munro = blue; COSMOS TTC = green; Cosmetics Inventory = red).](image)

Furthermore, the reactivity of the three datasets was compared by analysing their HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) energies distribution: the analysis showed that the Munro dataset has a slightly higher prevalence of reactive chemicals.

The chemical space of the datasets was also analysed by means of 3D plots, which visualise the overlap between the datasets in a 3D space defined by key descriptors. Figure 4.35 shows that the Munro and COSMOS datasets mostly overlap in the space defined by molar volume, solubility (log S) and dipole moment (polarity/reactivity). The COSMOS dataset tends to have more polar and water soluble structures.
Figure 4.35 3D plot of physicochemical space between the Munro and COSMOS TTC dataset. Munro = blue; COSMOS TTC = green.

The COSMOS Cosmetics Inventory covers a very diverse range of physicochemical properties. Comparison of the COSMOS TTC dataset with the Cosmetics Inventory in this way shows an overlap between the two datasets (Figures 4.36 and 4.37), indicating that the TTC dataset is representative of the chemical space of cosmetics in general.

Figure 4.36 3D plot of physicochemical space between the COSMOS Cosmetics Inventory and COSMOS TTC dataset. Cosmetics Inventory = red; COSMOS TTC = green.

When plotting the 3D space of the Cosmetics Inventory defined by log S, dipole moment, and molar volume, several chemical clusters emerged as illustrated in Figure 4.37. The combination
of water solubility, polarity/reactivity, and molecular size (volume) seems to separate well-known cosmetic ingredients including the quaternary ammonium alkyl chains, sugar polyols, ethoxylated alcohols, carboxylic esters, alkenes and retinoic acids clusters.

Figure 4.37 3D plot showing overlap in physicochemical space between the COSMOS Cosmetics Inventory (red) and COSMOS TTC dataset (blue).

The analysis was useful to identify and highlight some differences between the datasets. Compared with the COSMOS Cosmetics Inventory, the Munro dataset has slightly higher prevalence of reactive chemicals and a lower prevalence of larger, long linear chain structures.

The results of this preliminary analysis showed that the Munro dataset is broadly representative of the chemical space of cosmetics; the COSMOS TTC dataset, comprising repeated dose toxicity data for cosmetic ingredients, showed a good representation of the Cosmetics Inventory, both in terms of physicochemical property ranges, structural features and chemical use categories. Thus, this dataset is considered to be suitable for investigating the applicability of the TTC approach to cosmetics.

4.5.5 Development and Assessment of the Cell-Based Assay Model

Background

A combination of techniques is seen to be able to assess the toxicity of a certain compound, replacing, or at least reducing considerably, the need for the use of animals. These techniques
should include read-across, chemical categories, (quantitative) structure activity relationships ((Q)SAR), physiologically-based pharmacokinetic models (PBPK) and in vitro assays (DeJongh et al., 1999; Gubbels-van Hal et al., 2005). In addition, it is now becoming widely accepted that to progress on the understanding of toxic effects, understanding the toxic mechanism at a molecular level and how molecular changes relate to functional changes at higher levels of biological organisation (U.S. EPA, 2003) is crucial.

With regard to in vitro tests, the suggested refinements included the need to estimate the partitioning and bioavailability of the chemical in the assay to improve the methodology used to relate in vitro toxic concentrations to in vivo target tissue concentrations (DeJongh et al., 1999; Gubbels-van Hal et al., 2005). Following this approach, Kramer (2010) developed a fate model to simulate the partitioning of polycyclic aromatic hydrocarbons in cell-based assays considering the cells as another compartment.

The extrapolation from in vitro to in vivo organ level dose is complicated by the metabolic events that may occur in vivo, which may not be adequately accounted for by in vitro systems. In addition, in vitro systems will have different experimental factors, for instance the presence or absence of binding to cellular proteins, and also the distribution of the chemical into the culture medium, the apparatus or environment if volatile. Therefore, there is an urgent requirement to develop kinetics-type models such as PBPK models as well as for further development of metabolic simulators. It is apparent that being able to extrapolate from the concentration tested in vitro to a likely in vivo dose will be crucial for the successful implementation of many of the methods being developed in other projects of the SEURAT-1 Research Initiative. This will be vital to ensure the safety of cosmetic ingredients.

State of the art

The hazard assessment of a chemical has traditionally relied on animal models, with protocols that have been standardised over the years (OECD, 1993), and on the application of assessment factors (AFs) to take into account uncertainties associated with the extrapolation of animal model results to humans. However, the need to reduce and eventually replace the use of animals in toxicology testing is driving developments as the prediction of human in vivo toxic doses from concentrations that cause effects in vitro, with a minimum of intermediate animal testing. This implies the need to consider both toxicokinetics and toxicodynamics as important, if not essential, part in the risk assessment strategy (Adler et al., 2011).

COSMOS partner ‘French National Institute for Industrial Environment and Risks’ (INERIS) has already developed a PBPK model for humans based on a detailed compartmentalisation of the body. The model was calibrated with relationships describing the time evolution of physiological and anatomical parameters (Beaudouin et al., 2010). Kinetics can consequently be predicted for persons of different ages or for a given person along its lifetime, including pregnancy. Moreover, probability distributions were defined for key parameters related to
absorption, distribution, metabolism and excretion (ADME) to simulate the human variability and identify sensitive populations. This model will be used to predict the internal dosimetry of the chemicals of interest for long-term exposures in humans. It will also be the basis for the development of generic PBPK models for other species.

The overall strategy is to formulate an integrated modelling approach that will incorporate toxicological data from the corresponding levels:

- Dynamic Energy Budget (DEB) models of cell lines to correct *in vitro* data to make predictions of *in vivo* organ level toxicity by calculating internal concentrations.
- Dynamic models at each level to determine the temporal dynamics, distribution of the chemical in the different media and the rate limiting process.
- An *in silico* liver to enable detailed analysis of the metabolic aspects and to define the level of modelling detail needed to extrapolate from *in vitro* data to predict dose upon long-term exposure.
- Molecular Systems biology including metabolic and control networks of the corresponding cell line PBPK models.

The characterisation of the concentration that produces an effect (whether this is a perturbation to a molecular pathway or an apical toxic endpoint) is necessary at two levels; first for the *in vitro* experiments, since ‘nominal’ concentrations do not represent the real concentration experienced by the cell; and, second, in the extrapolation of the dose for human toxicity assessment, since to assess the hazard of a chemical compound, the true concentration experienced by cells within the target organ is required.

Approach

One possible way to solve both problems, and to be able to compare the same concentration values from *in vitro* and *in vivo* experiments, is to use biology / physiology-based toxicokinetic / toxicodynamic models at both levels. For *in vitro* experiments a model comprising the fate of a compound in the cell-based assay can be constructed, i.e., its partitioning between the plastic wall, serum proteins and lipids and potentially the compound’s dynamics within the cell; combined with a cell growth model and a toxic effects model. These coupled models allow for the simulation of the true concentrations causing perturbations in cells given the nominal concentrations applied in a microtitre plate well. An analogous approach *in vivo* is provided by physiologically-based toxicokinetics or pharmacokinetics models (PBTK or PBPK).

To this end, a process-based model able to predict the relevant concentrations and the dynamic behaviour in cell-based assays has been developed. In parallel, a model at the organ level (liver) and a whole organism (rat and human) PBTK model have been implemented,
and a Systems Biology model at molecular level for specific endpoints is under development. The coupling of these models should allow exploring the continuum toxic effects, to establish an interface between different levels in terms of data and results transferability, and finally to cover the different spatial and temporal scales involved in adverse outcomes.

Results

Based on in-house *in vitro* data from the HTS (High Throughput Screening) facility as well as data provided by US EPA and scientific publications, a process-based model able to simulate the dynamics of a chemical compound in cell-based assays has been developed. Specifically, the model calculates, based on the physicochemical properties of the compound, the dissolved (free) concentration, the concentration attached to the plastic, the concentration bound to proteins and lipids, the concentration in the headspace and finally the concentration inside the cell.

The model, illustrated in Figure 4.38, has the following components: (i) a fate and transport model; (ii) a cell growth and division model; (iii) a toxicodynamics model.

Figure 4.38 The cell-based assay model. a) Overview of the process included in the fate and transport model. b) Discrete cell stage-based model. c) Schematic representation of the chemical partitioning in the cellular compartments.
The solution of the ordinary differential and discrete equations of the model allows the calculation over time of the dissolved concentration of a chemical as well as the internal concentration inside the cells. To assess the performance of the model, in-house experiments, US EPA datasets and literature data have been used. For example, the influence of serum in cell-based assay experiments has been analysed using the data from Gülden et al. (2001) who found a linear relationship between the EC50 values and the serum level for several compounds. To compare with these results, the dissolved concentration at low serum level has been calculated. Subsequently, the value of this nominal concentration, which would produce the same dissolved concentration with increasing amount of serum in the medium, was calculated (see Figure 4.39). The model reproduces linear relationships and predicts the EC50 at high serum levels.

![Figure 4.39](relation_toxic_potency_of_p,p'-DDT_dieldrin_4-chlorophenol_and_pentachlorophenol-defi.png)

**Figure 4.39** Relation between toxic potency of p,p'-DDT, dieldrin, 4-chlorophenol and pentachlorophenol – defined as the nominal concentration that produces a dissolved phase concentration equivalent to that obtained at [S]0=1.81 10^{-2} mol/protein m^{-3} – and albumin concentration. Experimental data from Gülden et al. (2001).

The toxicokinetic model is based on the DEB (Dynamic Energy Budget) theory and assumes mortality proportional to the excess of internal concentration in comparison to a certain threshold. Figure 4.40 shows experimental and simulated data concerning the partitioning of PBDE-47 between the considered compartments. As it can be observed for PBDE-47 there are large differences between nominal and dissolved (free) concentrations. This is not the case for MeHg (data not shown). In addition, in the first case there is a considerable delay until internal cell concentrations reach the steady state, whereas for MeHg the process is faster.

The dynamics of the cell population has been analysed using US EPA data on Real-Time Cell Electronic Sensing (RT-CESTM). This approach yields a quantity called the Cell Index (CI), which, importantly for the current application, is linearly related to the number of cells.
(Xing et al., 2005). In addition, Figure 4.41 shows the results of a simulation using in-house cell line-based assays. The concentration-response curve was used to fit the toxicodynamic parameters of the model.

**Figure 4.40** Chemical partitioning of PBDE-47 in primary cultures of rat neocortical cells; in red experimental results from Mundy et al. (2004).

**Figure 4.41** Simulation of valproic acid in HepG2: a) Experimental and simulated concentration-response for an increasing concentration between 0 and $6 \times 10^{-2}$ M ($5 \times 10^{-3}$ M steps); b) Relative number of living cells ($N_t/N_0$); c) Simulated dissolved concentration; d) Simulated internal cellular concentration.
4.5.6 The KNIME Server: Extension of the KNIME platform for integration of COSMOS methods and models

Background and State of the Art

KNIME is the modular integration platform for the methods developed in the COSMOS project. 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. The KNIME technology integrates access to databases, data processing and analysis, as well as modelling approaches into flexible computational workflows that will be adaptable and form a set of building blocks allowing users to incorporate their own data and search existing data compilations.

KNIME provides a simple extension application programming interface 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 working areas.

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 is desirable that workflows can easily be shared by all groups during development and once they are usable for the public they should be easily accessible and usable even for non-experts in KNIME. The COSMOS computational workflows implemented in KNIME will be made publicly accessible and provide a transparent method for use in the safety assessment of cosmetics.

Approach

The work in COSMOS requires an enhancement of the KNIME integration platform with KNIME extensions allowing additional data sources and tools to be integrated, by means of (i) new implementation of nodes; (ii) assisting partners in collaboratively modelling their protocols as KNIME workflows; (iii) setup of an archival framework allowing reproducible execution of workflows, allowing sharing of methods and completing workflows via a central repository, and providing workflows to end-users via a simple (web-based) frontend.

KNIME Desktop offers the full functionality of KNIME regarding the creation and local usage of workflows. Since it is open source it can be used by anyone without any restrictions. However, in the context of a large research project with several groups it is essential that the developed methods and extensions as well as the workflows can easily be shared. 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 stable pre-built workflows are available to be used outside the COSMOS project they should be accessible in an as simple as possible way without the need to install several programmes.
The solution to these issues is 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. Workflows can also be executed automatically at certain points in time e.g. to perform regular maintenance tasks.

Results

The public application programming interface (API) of the KNIME platform has been documented and partners have been trained in programming against the API. In addition a prototype of the KNIME Server has been implemented which is a two-part concept: an extension to the KNIME Desktop which allows access to the server (essentially the workflow repository) and the server itself.

The desktop side of the KNIME Server is represented by the so-called KNIME Explorer, which is an additional view inside the graphical user interface that shows several workflow repositories, e.g. the local workspace, a shared drive or one or more KNIME servers (see Figure 4.42a). 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 fetched afterwards. Also fine-grained read, write, and execute permissions on 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. Figure 4.42b shows an example with the server repository containing all available workflows on the left side. The right side shows the adjustable settings for the selected workflow. In the example the user can define the number of clusters that should be created while clustering a set of chemical compounds. It is also possible to upload complete files as input to workflows through the web portal. Once the workflow has been fully executed on the server, the user can retrieve the results. This can either be simple numbers, data files or even sophisticated reports containing tables and diagrams in common formats such as PDF or PowerPoint.

The server not only allows for the sharing of complete workflows but is also able to host so-called metanode templates. A meta-node is kind of a sub-workflow that encapsulates a group of nodes that perform a common task together. This is quite useful in cases where there is no single node available but the results can also be computed with a sequence of nodes. Using the KNIME Server through the explorer it is possible to store such a metanode as a template on the server. This template can then be used in other workflows (by other users) similar to normal nodes. Furthermore, if the copy in the workflow is linked to the template on the server,
it can automatically be updated once the template has been changed. The usefulness of this concept is obvious: if a project partner has developed a protocol to perform certain common tasks (e.g. computation of several biochemical descriptors) other groups can easily re-use and build upon the metanode in their workflows. Moreover the developers can easily update the template (e.g. fix errors, improve the model, etc.) and the other groups can immediately benefit from it.

![Figure 4.42](image)

**Figure 4.42** a) The KNIME Explorer offering a unified view on several storage locations for KNIME workflows. b) The KNIME WebPortal showing several workflows on the KNIME Server.

### 4.5.7 Innovation

The original contributions of the COSMOS database framework include on one hand the main data schema for the COSMOS database, built on a 3-tier architecture model, and on the other hand the collaborative framework for users to manage own and repository object data, which are both foundations of a data governance framework. Data governance at this stage is implemented in a bottom-up approach, by considering the data quality check and also collaborative work environment for COSMOS users. The Social Network model for COSMOS users allows a registered user to define a circle of his/her friends and interests groups, in order to provide access to their own data and/or exchange data and models between each other. Meanwhile, access will be granted at chemical compound level and stored data models by a set of flags. In this way users could further develop specific dataset objects which will be part of KNIME workflows in an integrated way.

By employing the cheminformatics methodology, the first comprehensive COSMOS Cosmetics
Inventory and the new COSMOS TTC dataset were compiled as databases. The nature of a cheminformatics database linked to toxicity endpoint data will allow for the delivery of the new TTC dataset more transparently with rules documented within the database. The use of physicochemical properties to differentiate the datasets will also assist in oral-to-dermal exposure extrapolation needed in the TTC assessment of cosmetic ingredients. A new subgraph representation method, CSRML, will also be applied in more detail to classify compounds into the structural categories of the TTC dataset. The structural knowledge has been implemented either as SMARTS or CSRML using the KNIME technology or MOSES applications (Molecular Networks). Altogether, this innovation will eventually enable the deployment of an innovative TTC assessment tool.

A process-based model able to predict the relevant concentrations and the dynamic behaviour in cell-based assays was developed. First results suggest that the approach may open a new way of analysing particular types of experiments as well as a new approach towards in vitro concentration to in vivo dose extrapolation (IVIVE) by comparing the same values in all the systems irrespective of the nominal concentrations.

4.5.8 Cross-cluster Cooperation

COSMOS compiled the Cosmetics Inventory v1.0 as the first comprehensive compilation of cosmetic ingredients. It is provided as a database and can thus be shared with the other projects of the SEURAT-1 Research Initiative. COSMOS interacted with ToxBank for the setup of the COSMOS database and relevant API as well as on chemical selection and also participated in the ToxBank Data Analysis Working Group (DAWG).

The PBPK and in vitro to in vivo extrapolation COSMOS working area has developed a first case study in multi-scale modelling with acetaminophen, in single and multiple dose situations with data from DETECTIVE. Another line of work includes coupling, with an in silico model of the liver, the internal metabolism of the hepatocytes (NOTOX) with a simple 3D model of the liver and predict toxic effects distributed in space and time inside the organ.

A first version of the cell-based assay model using an open source platform (KNIME/R) is being tested. A prototype version will be distributed between interested partners in the SEURAT-1 consortium so they can characterise, analyse and simulate the dynamics of their cell-based assays experiments. It is expected that the set of complete models will improve the results of in vitro - in vivo extrapolation and, hence, reduce or replace animal experiments.

The TTC approach can be a practical viable alternative to reduce animal testing and thus can play an important role in the 7th Amendment mandate expected in 2013. COSMOS partners include ILSI Europe and their expert working groups on the extension of the current TTC approach to cosmetic ingredients and the evaluation of oral-to-dermal extrapolation. Many of
the experts in this group represent the members of Cosmetics Europe.

Other cross-cutting activities from COSMOS include the discussion of Adverse Outcome Pathways, e.g. within the COSMOS-chaired SEURAT-1 Mode of Action Working Group.

4.5.9 **Expected Progress within the Second Year**

The COSMOS Project has a number of key goals with defined plans to achieve them. With regard to data collation, curation and sharing the long-term goal is to provide a database platform that will succeed COSMOS. In the second year of the project, there will be the deployment of the chemical and toxicological data management prototype to the SEURAT-1 consortium. This repository will allow the exchange and collection of data at testing stages in a consistent way, supported by data curation strategies, during year 2. In the medium term, research on the requirements and impact of a data governance framework for the COSMOS repository will be provided and conclusions published. This will lead to a functional database by the end of the project.

The COSMOS Project will provide working TTC models and software to provide a basis for the incorporation of information from Adverse Outcome Pathways (AOPs). The ultimate goal beyond the SEURAT-1 Research Initiative is to incorporate a mechanistic rationale into Cramer Tree and TTC decision schemes and reflect the paradigm shift toward the regulatory science of the 21st century. In the second year, COSMOS will deliver a quality-controlled COSMOS TTC database with oral repeated dose NOAEL values, also documenting study inclusion criteria and the rules used to determine NOAELs based on data from various sources. The data content will be expanded with additional regulatory data harvested from US FDA and EC SCCS sources. The thresholds for the Cramer Classes will be reported based on the cumulative distribution analysis of oral NOAEL values. Furthermore, a set of bioavailability data including skin penetration and Caco-2 cell permeability will be provided. The oral-to-dermal extrapolation will be evaluated by penetration models and, in some selected cases, by PBPK modelling. Within the second year, COSMOS plans to deliver a practical opinion on the use of the TTC approach to address the product safety of cosmetic ingredients. The COSMOS TTC database can provide the basis for the thresholds for cosmetic ingredients, considering also the oral-to-dermal extrapolation. Within the scope of the SEURAT-1 Research Initiative, COSMOS will deploy a software tool of the TTC database and a workflow implemented in KNIME.

COSMOS will ultimately provide freely available computational tools for prediction of toxicity (e.g. read-across, QSAR etc.) in addition to an assessment of chemical space. The COSMOS project will also embrace new ways of thinking, such as the development of AOPs. In year 2, these activities will be progressed by a thorough analysis of chemical space. In addition, more effort will be made in the development of models. Following the characterisation of chemical
space of the Cosmetics Inventory, optimisation of in silico methods, such as (Q)SAR, grouping and read-across, for the purpose of predicting long-term toxicity of cosmetic ingredients. 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 such as Cramer rules as a grouping scheme. Furthermore, the information on the biological profile of the chemicals will be considered in the similarity analysis. QSAR models and expert systems predicting the chronic toxicity endpoints that ‘drive’ the TTC thresholds will be searched for suitable groups of the chemicals of the Cosmetics inventory. A key activity for COSMOS is the definition of the molecular initiating event and the possibility of using this for chemical grouping and read-across. This will be linked to the broader work within the SEURAT-1 Research Initiative, in order to develop AOPs.

Allowing for effective extrapolation of the effects of an in vitro concentration into a dose in vivo is also an important goal of COSMOS. The main priority in this area for the second project year is to implement the PBTK model and to couple it with the cell-based assay model. In addition, the 3D liver model will be interfaced to consider spatial inhomogeneities and they will be assessed to know when it is necessary to take them into account. Finally, in order to move to a more descriptive approach using a toxicity pathways and MOA (Mode of Action) framework, systems biology models at a molecular level will also be developed.

All activities in COSMOS will be supported by the KNIME software, resulting in openly available and transparent workflows. With the increasing use of the KNIME Server prototype, additional functionality or usage improvements will be required in the second year. In addition, archiving functionality has not been implemented yet, but will become more important once complex workflows are established and being used for progressing work throughout the project. A mechanism for versioning and archival is necessary in order to guarantee consistent and reproducible results.

4.5.10 Future Perspectives

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

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

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

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

Models for toxicodynamics and toxicokinetics will be developed within COSMOS and will form the foundation of research beyond SEURAT-1. It is already widely acknowledged there is a great need to develop further the capabilities for in vitro – in vivo extrapolation. This will allow for the better application of results from cell-based assays to perform human safety assessment. Amongst the strategic requirements for SEURAT-2 will be kinetics modelling (e.g. through Physiologically-Based PharmacoKinetic (PBPK) models); a better understanding of the effect of the properties of the test systems (e.g. sorption) and chemicals (e.g. volatility, stability) relating to extrapolation; and metabolism, its modelling and prediction. Integrated efforts within COSMOS will also result in workflows for toxicity prediction. A finding from COSMOS will undoubtedly be that there is no simple computational method to predict organ level toxicity. Therefore, within SEURAT-2 there is a strategic requirement to develop and utilise open and transparent platforms, such as KNIME to capture and implement modelling processes. Ultimately this will lead to a platform supporting data capture, storage and retrieval, links of chemistry to pathways through AOPs and open and flexible modelling for relevant endpoints to evaluate safety of chemicals to humans.
References


Further recent key references of project members related to the field of research


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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 forthcoming complete ban on animal testing in Europe for cosmetic products and the associated need to develop better prediction models in this area (Hartung et al., 2011). We use a systems biology approach with interlinked experimental and computational platforms. The models will be multi-scale, from molecular to cellular and tissue levels. Since testing on the target organism, human, is not possible, human organotypic cultures have the potential to allow repeatable, transferable testing of highest possible relevance. Multi-scale models shall eventually incorporate the obtained experimental data to predict human long-term toxicity. We can expect that in the near future cellular systems derived from human stem cells will be preferred for testing purposes. Ultimately, it will be necessary to collect experimental data from all relevant tissues including the interactions between tissues and organs. Since liver plays a central role in metabolism, both concerning normal physiological function as well as xenobiotic metabolism, we selected hepatic cultures for the NOTOX project. Since human hepatic cells derived from stem cells are not yet readily available with sufficient functionality, we selected HepaRG cells, a hepatocarcinoma cell line for the starting phase of NOTOX. This cell line has been shown to be the closest to primary human cells in terms of metabolism of xenobiotics expressing important cytochrome P450 enzymes (CYPs) at high levels (Kanebratt & Andersson, 2008a; 2008b). For validation purposes and for the development of new techniques, we also use the well-established HepG2 cell line and primary human hepatocytes. In these test systems, viability and physiological toxicity response parameters (‘-omics’) will be monitored together with structural characteristics in parallel. Large-scale network models of regulatory and metabolic pathways and cellular systems together with bioinformatics integration of human
and cross-species literature data will lead to reliable toxicity prediction. 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.

The experimental platform focuses on long-term response of human cell lines and primary cells (preferentially derived from stem cell lines, cultured particularly as organotypic 3D cultures), and their detailed structural and functional characterization. The response will be monitored using a combination of cutting-edge ‘-omics’ technologies, e.g. in-depth dynamic metabolic flux analysis using 13C labelling and 3D cryo-electron tomography. The initial focus will be on human liver cells, since liver is the most relevant systemic toxicity-target organ. Chosen hepatic cells will be used in long-term membrane, spheroid and sandwich cultures. As available, human target cells and organ simulating devices from other projects (see project descriptions of SCR&Tox and HeMiBio) of the SEURAT-1 Research Initiative will be implemented.

The organotypic model systems in a long-term setup have been exposed to repeated low doses of selected test compounds with known toxicity and future industrial relevance. These compounds will be selected in close collaboration with the integrated data analysis and servicing project of SEURAT-1 (ToxBank). The physiological effects of test compounds on the test systems will be monitored by determining ‘-omics’ data (epigenomics, transcriptomics, proteomics, metabolomics, fluxomics) at various time points. Large-scale modelling of regulatory and metabolic pathways will simulate toxic responses. Design of experiments will be assisted by toxicophysiology data obtained from literature and databanks as well as from computer simulations of in silico cells.

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

Effects upon long-term exposure to test compounds as monitored and measured by abovementioned technologies will be analysed by bioinformatics methods. Data from databases, literature, experiments and simulation will be integrated through bioinformatics tools to create a knowledge base for quantitative understanding of toxicity response pathways and regulatory networks at the molecular level. These data will provide the base for prediction models.

Individual biological pathways will be described by corresponding mathematical models that can be integrated into mathematical tissue models and eventually into a large-scale whole system mathematical model. Since such large-scale computational systems biology models often comprise a large set of equations and include hundreds of thousands and even millions of data points, strategies will be developed using state-of-the-art multi-core and grid computing for analysis and exploration of these models.
The major objectives of NOTOX in its first phase are:

- 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 characterization of 3D organotypic cultures after long-term repeated dose exposure to selected test compounds (individual epigenetic chromosomal profiling, 3D-EM-tomography, 3D-topographic analysis and modelling, bioinformatic characterization).

- Development of predictive causal computer models aimed at entering pre-validation as guided by ToxBank and as defined by ECVAM.

4.6.2 Cellular Systems and Cultivation Techniques

State of the Art

Human cellular systems for long-term toxicity testing: One of the major obstacles in the creation of human cell-based long-term test systems is the lack of reliable availability of cellular systems exhibiting stable and reproducible long-term viability and functionality. This is a necessary prerequisite for such type of testing. In the NOTOX project, the initial focus will be human liver cells and cell lines (e.g. primary cells and HepaRG cells in organotypic cultures), as the liver is the most relevant organ when dealing with toxicity because of its enormous metabolic diversity comprising of CYPs, conjugating enzymes and transporters with typical polarity of the hepatocytes. Compound metabolism is linked to the carbon and energy metabolism of the cells. Toxic effects and changes in compound metabolism are, therefore, reflected in the changes in the cellular metabolism. Primary human hepatocytes are widely used as an in vitro cell model in drug discovery, but their use has limitations since they rapidly lose metabolic functions when cultured and show individual specific variations in addition to their limited availability. Cell lines can be used with evident advantages, such as wide availability and stable phenotype. However, the majority of the human hepatic cell lines most commonly used today contain only low levels of enzymes and transporters necessary for the hepatotoxic drug actions (Donato et al., 2008). Therefore, there is a need to develop hepatic cell lines that functionally resemble hepatocytes in vivo. Moreover, other cell types present in the liver, such as Kupffer cells, monocytes/macrophages and stellate cells, also play an important role in hepatotoxicity (Michael et al., 1999; Muriel and Escobar, 2003).

The HepaRG cell line is derived from a human liver carcinoma (Gripon et al., 2002). HepaRG
cells possess the unique property to differentiate in vitro and to express various liver functions, including CYPs, phase II enzymes, transporters and nuclear receptors at levels comparable to those found in primary hepatocytes and are responsive to prototypical inducers, suggesting that they could represent a surrogate to the latter in drug metabolism and toxicity studies (Aninat et al., 2006; Le Vee et al., 2006; Guillouzo et al., 2007; Josse et al., 2008; Kanebratt and Andersson, 2008b; Turpeinen et al., 2009). Moreover, some evidence has been provided that HepaRG cells can retain relatively stable expression and activities of CYPs for several weeks at confluence (Josse et al., 2008; Kanebratt and Andersson, 2008a), giving the possibility for long-term toxicity studies.

Cultivation systems: An interesting in vitro system for studying drug-induced hepatotoxicity is the bioreactor system originally developed by Gerlach et al. (2003) and then miniaturised at various scales (Schmelzer et al., 2009; Zeilinger et al., 2011), where it will be possible to study metabolism and toxicity because the cells maintain their phenotype for very long times (Mueller et al., 2011b). 3D culture of cells can be achieved by sandwich cultures using appropriate extracellular matrix (Godoy et al., 2009). Using a serum-free 2D system, it has been shown that long-term culture of human hepatocytes for more than four weeks is possible and that repeated exposure rounds are possible and result in reproducible cycles of compromised function (such as reduced albumin and ammonia secretion), followed by complete recovery when the test compound is removed from the culture medium (Ulrich et al., 2007). Spheroid cultures have been used for a long time, but recently Brophy et al. (2009) described a successful formation of rat hepatocyte spheroids in rocked culture that showed stable expression of more than 80% of 242 liver-related genes, including those of albumin synthesis, urea cycle, phase I and II metabolic enzymes, and clotting factors. HepG2 cells have been cultured on a gyratory shaker to produce spheroids (May et al., 2009). A promising new technique is presented by InSphero (Zurich) allowing the formation of scaffold free spheroids in special microtiter plates. In another approach, the group of Kajiwara reported a co-culture of rat hepatocytes and human umbilical vein endothelial cells (HUVECs) in spheroids allowing a vascularised liver organoid with retention of tissue-like structure in hollow fibres (Inamori et al., 2009).

Approach

Organotypic cultures mimic physiological conditions more accurately by inducing cell-cell and cell-matrix interactions, which are not present in 2D monolayer cultures. A recent study by NOTOX partner ‘Saarland University’ showed that HepG2 spheroids are a suitable model for toxicity assessment, particularly in case of anti-cancer drugs (Mueller et al. 2011a). The NOTOX project will evaluate the effects of long-term repeated dose exposure to test compounds by using different ‘-omics’ technologies. It was already shown that repeated dose exposure using functional in vitro cultures can effectively support the use of in vitro alternative methods (Mueller et al., 2012).
Results

During the first year of the NOTOX project, we established the spheroid cultivation system supported by InSphero and successfully applied it to HepG2, primary human hepatocytes and HepaRG cells. HepaRG cells formed compact spheroids between days 2–3 after initial seeding which did not grow in size since differentiated HepaRG cells do not proliferate under used conditions (Figure 4.43).

**Figure 4.43** Formation of HepaRG spheroids (initial cell numbers 500 – 8000) during 6 days of cultivation (d0 = seeding). Scale bars = 200 µm.

The established system allows accurate adjustment of the spheroid size, medium refreshment and spheroid harvesting and can, therefore, be used for the analysis of intra- and extracellular parameter. The NOTOX partner ‘Biopredic International’ investigated the morphology of HepG2 and HepaRG spheroids by applying cross- and longitudinal sectional hematoxylin- and eosin staining (Figure 4.44).

**Figure 4.44** Cross- and longitudinal sectional hematoxylin and eosin staining on HepG2 and HepaRG spheroids. Scale bar = 300 µm.
It was observed that the spheroids are disc-like in morphology. Cells are homogeneously distributed across spheroids and, in contrast to monolayer cultures, HepaRG spheroids are predominantly composed of hepatocyte-like cells. At the tested growth conditions, the cells inside of the large HepaRG spheroids appear to be necrotic probably due to nutrients and oxygen diffusion limitations. A 3D hollow fiber system, based on recent studies by the Kajiwara group (Mizumoto et al., 2008), was developed and established in the laboratories of NOTOX partner ‘Saarland University’. HepG2 cells were maintained in a 3D tissue like structure within the hollow fibers and serum free cultivation (more than 4 weeks) could be performed giving the possibility for repeated dose testing of test compounds and the assessment of chronic effects. This 3D hollow fiber system will be applied to HepaRG cells in the next future as functional 3D cell culture system for long term repeated dose testing.

In addition, the NOTOX project partners ‘Karolinska Insitute’ and ‘Saarland University’ will also use a 3D hollow fibre bioreactor system developed by Gerlach et al. (2003) for cultivation of human liver cells for prolonged periods (Mueller et al., 2011b), in order to study the mechanisms behind drug-induced liver toxicity and to identify early biomarkers that can predict such reactions. Moreover, this bioreactor system will be applied to differentiate hESC and iPSC to hepatocytes and non-parenchymal liver cells to form intact hepatic structures aimed at studying the long-term toxicity at ‘Karolinska Insitute’. Differentiated HepaRG cells in co-cultures with lymphocytes and Kupffer cells will be also used as well as stem cells for characterization of toxicological and carcinogenic properties of chemicals.

Biochemical and clinical parameters of the cells in the bioreactor are analyzed in order to characterize the conditions/performance of the cells in the bioreactor. NOTOX partner ‘Karolinska Insitute’ cultivated primary liver cells (obtained from collaborators at Huddinge University Hospital) using small 0.5 ml bioreactors, while maintaining liver-like structures, including cells positive for CK19 and CD68 and bile ductile-like structures. Cytochrome P450 activity remained ongoing for 2 weeks, in particular for CYP1A2 and CYP2C9 catalyzed reactions. One of the three bioreactors displayed identical activity as on day one. In addition, partners from the ‘Karolinska Insitute’ have recently applied the novel type of bioreactors with oxygen sensors and units which are more easily removed for immunohistochemistry analysis.

For toxicity testing, primary cells maintained in the bioreactor have been exposed to troglitazone and acetaminophen. Levels of transferases, urea, glucose and other clinical parameters were monitored. It is necessary to determine the actual drug concentration in the media, since the bioreactors sometimes adsorb large amounts of drugs, in particular the basic drugs. In some bioreactors, the recovery of cells has not been optimal when harvested 2-3 weeks after inoculation. The conditions are further optimised for the detection of relevant toxicity during prolonged periods. In the longer perspective, NOTOX will focus on running the bioreactor units from different patients, who are susceptible or not, for drug-induced toxicity as outlined in the Figure 4.45.
Figure 4.45 Scheme depicting the procedure to obtain 3D-bioreactor structures from different human livers, some from patients susceptible for toxicity and other from controls. Isolated hepatocytes can be dedifferentiated into progenitor and later to iPS cells, which in turn are converted by transient gene expression into hepatocytes and non-parenchymal liver cells, which are combined into 3D liver bioreactor structures that can be used for in vitro toxicity assays in an integrated relevant hepatic model.

4.6.3 ‘-omics’ Technologies and Toxicity

State of the Art

In NOTOX, a set of ‘-omics’ techniques will be applied. Earlier studies showed massive alterations in gene expression patterns upon comparing the in vitro systems to the in vivo situation (Hewitt et al., 2007), but considerable improvement was shown in advanced systems (Schug et al., 2008; Godoy et al., 2009). The revolutionary developments in sequencing greatly stimulate epigenomic research. Epigenetic marks are highly correlated with all levels of gene regulation, are very stable and provide a kind of ‘cellular memory’. In NOTOX, a first comparative epigenetic and transcriptomic analysis of primary cells and HepaRG as well as HepG2 cells, is planned. Proteomics provides information closer to cellular activity and differential proteins can be identified and validated as biomarkers (Miguet et al., 2009). Techniques for extracellular and intracellular proteomics are adapted in the first phase of NOTOX. Toxic effects and even sub-toxic effects are manifested in the metabolic changes, seen in both the fluxome and metabolome (Strigun et al., 2011a-b; Strigun et al., 2012). Fluxomics can provide very detailed information on cellular metabolism even on inter-compartment fluxes (Niklas et al., 2011a-d).
Approach

Extracellular ‘-omics’ data, particularly on the metabolome and proteome, will be collected at regular intervals. Dynamic experiments with high frequency measurement of intracellular metabolites after $^{13}$C labelling will assist configuring and parameterising metabolic models. Intracellular ‘-omics’ data, particularly transcriptomic and epigenetic data, will be measured at selected time points.

**Metabolomics / Fluxomics:** Metabolomics, the qualitative and quantitative analyses of metabolites in a certain biological sample, is a sensitive tool to detect test compound-induced effects and can, therefore, contribute to the understanding of adverse reactions and toxic effects (*Strigun et al., 2011a-c*). A recent study by the NOTOX partner ‘Saarland University’ shows significant effects of long-term repeated dose exposure to diclofenac on the metabolome of primary human hepatocytes even at clinically relevant concentrations (*Mueller et al., 2012*). This established method will be applied to the investigation of acetaminophen-induced metabolic alterations on HepaRG cells in 2D monolayer and 3D organotypic cultures. Moreover, stationary and dynamic metabolic flux analysis will be carried out to analyze the effects of test compounds on extra- and intracellular reaction rates. A recently published metabolic network model (*Niklas et al., 2009*), based on the method of metabolite balancing, was already adapted and applied to HepaRG cells by NOTOX partner ‘Saarland University’ for the investigation of acetaminophen-induced metabolic effects.

**Epigenomics:** The NOTOX partners ‘Saarland University’, ‘Karolinska Insitute’ and ‘Weizman Institute of Science’ have a strong expertise in epigenomic profiling. NOTOX, in particular, will apply the DNA-methylation analysis to monitor toxic effects on the cellular epigenome (toxicoepigenomics). The potential of primary human hepatocytes as a testing model for toxicological testing is limited by several factors, i.e. availability of these cells and altered hepatic properties under standard culture conditions. The recently introduced HepaRG cell line is a promising alternative to primary cells. It maintains the activity of many liver-specific factors such as CYP450 activity in a 2D-culture, unlike the widely used HepG2 cell line. Recent studies support this idea and postulate a higher degree of transcriptional similarity between HepaRG and primary human hepatocytes, if HepG2 is taken as a reference (*Hart et al. 2010, Jennen et al., 2010*).

**Proteomics:** Toxicity is also reflected in alterations of protein expression. Proteomics is widely used in pharmaceutical research to detect and predict toxicity *in vitro and in vivo* (*Dowling and Clynes, 2011*). However, in standard mammalian *in vitro* cultures, fetal calf serum (FCS)
is used for cell adherence and maintenance. This supplement is an obstacle in extracellular proteome analysis because proteins of bovine origin hamper identification of proteins secreted by the cells of interest.

Results

Epigenomics: Starting from (a rather limited) \textit{a priori} knowledge on the common molecular feature, the \textbf{NOTOX} partner ‘Saarland University’ attempted to elucidate the mutual epigenetic relationships between primary human hepatocytes and possible \textit{in vitro} hepatocyte models, as well as their association to the observed differences at the transcriptional level. DNA methylation was selected as a target epigenetic mark due to its relative stability and accessibility. A genome-wide DNA methylation and RNA expression analysis of HepG2 and HepaRG cell lines was performed and compared to freshly isolated primary human hepatocytes and 7 days cultured primary human hepatocytes. The analysis used Illumina bead array platforms covering approx. 450,000 CpG positions (~2% of all genomic CpGs) and 43,000 transcripts including a number of non-coding RNAs, respectively.

Comparative transcription profiling is in line with previous observations from the literature, showing that HepaRG cells share stronger similarity to fresh and cultivated primary human hepatocytes samples as compared to HepG2 cells (Figure 4.46). Differential expression analysis was conducted in two settings. First, each cell type was characterized with several hundred to a couple of thousand specific significantly changed transcripts. Applying a stringent effect size threshold – an expression change of an order of magnitude or higher – the set of differential transcripts to more than two hundred of high-confidence targets was refined. Intriguingly, the dominating bulk of the differential transcripts can be classified as being predominantly or specifically expressed in the HepG2 cells. Thus, it can be concluded that the most pronounced transcriptional differences between the compared cell types are due to the specific expression regulation in HepG2 cells. Second, the matched comparison of the PHH samples before and after cultivation allowed identifying a relatively small number of differential transcripts. This finding suggests that short-term cultivation has only moderate effects on DNA methylation.

In contrast, the variability of DNA methylation profiles was attributed to a much higher extent to the cell type, both in the exploratory and in the differential analysis. If clustered, the DNA methylation profiles formed compact domains with very low intra-class dispersion (Figure 4.46B). In cell-type differential analysis the numbers of significantly different CpGs reached the order of $10^4$ for each class, whereas the cultivation did not cause genome-wide significant DNA methylation changes in the analyzed samples.
The NOTOX partner ‘Weizman Institute of Science’ possesses a strong bioinformatics background focused on developing methodology for understanding and using DNA methylation patterns in somatic tissues, with a particular emphasis on distinguishing between different loci and predicting the behavior of DNA methylation in different genomic contexts, as well as on studying DNA methylation heterogeneity within cell populations. These works form the foundation for the subsequent development of DNA methylation markers for toxicity, as they allow selecting loci and interpreting DNA methylation patterns occurring therein in a significantly enhanced computational framework.

Mammalian CpG islands are the key epigenomic elements that were first characterized experimentally as genomic fractions with low levels of DNA methylation. Currently, CpG islands are defined based on their genomic sequences alone. WIS developed evolutionary models to show that several distinct evolutionary processes generate and maintain CpG islands. One central evolutionary regime resulting in enriched CpG content is driven by the low levels of DNA methylation and consequentially low rates of CpG deamination. Another major force forming CpG islands is a biased gene conversion that stabilises constitutively methylated CpG islands by balancing rapid deamination with CpG fixation. Importantly, evolutionary analysis and population genetics data suggest that selection for high CpG content is not a significant factor contributing to conservation of CpGs in differentially methylated regions.

Proteomics: The liver cells produce serum proteins such as carrier-proteins (albumin, transferrin, haptoglobin), protease-inhibitors (antitrypsin) or complement factors. These proteins show a high degree of homology between different species. For example, human and bovine serum albumin share more than 76 % of their amino-acid sequences. Because
mass-spectrometry (MS) based protein identification relies on determination of amino-acid sequences of peptides which are subsequently assigned to a specific protein, this approach can hardly distinguish between human or bovine proteins in the supernatant. The NOTOX partner ‘Centre National de la Recherche Scientifique’ identified the subcellular location of proteins in a monolayer 2D culture of primary human hepatocytes at serum-free conditions by using modern state-of-the art LC/MS techniques (see Figure 4.47).

![Figure 4.47 Distribution of subcellular locations of the identified proteins as described in the Swiss Prot database. A total of 126 proteins were identified by LC-MALDI-MS/MS analysis of a tryptic digest of the extracellular proteome of primary human hepatocytes in standard monolayer culture. Samples were taken after 48h of serum-free cultivation. ER = endoplasmic reticulum.](image)

Almost 30% of identified proteins originated from intracellular compartments indicating cell death in the culture. The use of serum-free HepaRG cultivation medium and organotypic cultures developed by NOTOX partners will be advantageous for further proteomic analyses because these techniques not only avoid serum for cultivation but also closely resemble the in vivo situation of a real organ ensuring transferability to situations found in humans.

For modern state-of-the art mass spectrometry, it is mandatory to interpret the huge amounts of obtained data using exponentially growing protein databases. Today, a high calculation power is required in proteomics. In addition, the use of more than one search algorithm (at least one of them being open-source) is highly recommended in order to improve confidence in MS data interpretations. The identification of the most relevant information in the huge protein lists that are generated after MS data interpretation is time-consuming and proteomics has to turn into functional proteomics. Within the NOTOX project, the partner ‘Centre National de la Recherche Scientifique’ has developed a software pipeline called MSDA (Mass Spectrometry Data Analysis; msda.unistra.fr). It allows any required protein database to be created and customised from public databases (e.g. NCBInr, UniProtKB). It is also dedicated to search mass spectra using an open-source algorithm (OMSSA) and to automatically extract functional
annotations (gene ontologies) for all identified proteins. The functional annotation tool will soon be reinforced, with the possibility to automatically extract annotations from the KEGG database, build graphs bearing functional relationships between the identified proteins and estimate enrichment in one or another annotation within a huge set of MS data. MSDA has been adapted for use on a computer grid, thus giving access to calculation speeds 100-1000 times higher than before.

**Transcriptomics:** In contrast to primary hepatocytes, stem cells derived from the same source are reproducible and have the same characteristics. NOTOX partner KI has taken part in a study aimed to elucidate whether analysis of differential gene expression could be used to predict the type of toxicity. The usage of a hESC derived hepatocyte-like *in vitro* system (hES-Hep) was thus evaluated (Brolen et al., 2010) with a panel of fifteen chemicals which are classified as non-carcinogens, genotoxic carcinogens and non-genotoxic carcinogens, incorporating whole-genome transcriptome responses measured with Affymetrix microarrays. An analysis of a variance model that identified 592 genes was highly discriminative for the panel of chemicals and, thus, their effect on the transcriptome could be used to differentiate between non-genotoxic carcinogens, genotoxic carcinogens and non-carcinogens (Yildirimman et al. 2011).

**4.6.4 Structural Changes**

**State of the Art**

Histological observations have a very long tradition in toxicology. Modern methods of structural investigations on tissue, cellular and even supra-molecular levels open up new opportunities in this field. New optical imaging methods are rapidly emerging and will be intensively applied for studying 2D and 3D cultures. Methods of ultra-resolution cryo-electron tomography, not requiring conventional fixatives, dehydration and stains, permit creating maps of individual macromolecular complexes (Pierson et al., 2011). Such methods are further developed in the first phase of NOTOX as the new algorithms promise significantly improved resolution and contrast of such pictures (Xu et al. 2010, Agulleiro and Fernandez 2011, Herman and Davidi 2008), resulting in unprecedented structural characterization capabilities in near-live conditions.

**Approach**

Toxic compounds can induce structural changes not only on supracellular structure and morphology of tissues or organs, but also on sub-cellular, macromolecular levels. 3D-structures of the organotypic cultures will be studied using 3D cryo-electron tomography and light and confocal microscopy.
The NOTOX partner ‘Netherlands Cancer Institute’ has a strong expertise in cryo-electron tomography of vitreous section (CETOVIS). NOTOX is planning to provide information on structural modification induced by the test compounds in a cellular environment. The first step of CETOVIS is to HPF (High pressure freeze) the specimen of interest, preserving the ultra-structure of the cell. This approach allows visualisation of 2D sections of cells and tissue under condition, as close as possible, to the physiological environment. These sections may be electrically charged to ensure adherence to the grid that supports them. It is subsequently possible to tilt the grid under the electron beam of the microscope and collect a series of images at a different tilt angle of the specimen: the tomogram. Merging together the 2D images, a 3D representation of the specimen was assembled. This approach allows obtaining 3D structure of macromolecular complexes within the cell. Structures of molecular machineries in the cells such as GroEL, ribosomes, DNA polymerases and ATP-ases have already been described. Our aim is to go one step forward, i.e. observing 3D modification of the complexes induced by chemical compounds.

Results

In a recent study carried out by the NOTOX partner ‘Saarland University’ (Mueller et al. 2011a), the toxicity of the test compound tamoxifen was investigated and its effects on the structure and morphology of HepG2 spheroids were recorded. Tamoxifen clearly induced structural alterations in spheroid morphology assessed by live-dead staining and microscopic monitoring. Tamoxifen clearly induced structural alterations in spheroid morphology (Figure 4.48). Toxicity was observed at 50 μM (in the range of EC50). Exposure to 100 μM tamoxifen (24h) induced spheroid rupture and cell death on spheroid surface. Viability and morphological experimental data will further be explored by modeling groups within the NOTOX consortium to gain detailed understanding of the test compound effects on organotypic cultures.

**Figure 4.48** Morphology and viability of HepG2 organotypic cultures after 24h exposure to tamoxifen. Morphology: a) untreated control b) 50 μM tamoxifen c) 100 μM tamoxifen; Live/dead staining (FDA/PI) in d) untreated control e) 50 μM tamoxifen f) 100 μM tamoxifen. Scale bars = 200 μm.
The NOTOX partners ‘German Research Centre for Artificial Intelligence’ and ‘Netherlands Cancer Institute’ are working together on developing improved computational methods for the 3D cryo-electron tomography reconstruction and analysis. A novel fully 3D reconstruction framework ETtention targeted at high-performance computing using both multi-core CPUs and GPUs with support for advanced a priori regularisations, noise models, and improved sampling, has been developed. Within the framework, a block-iterative reconstruction method (SART) with very early termination and strong under-relaxation has been implemented. Figure 4.49 shows a slice from a 3D tomography reconstruction of a 50 nm thick vitreous section using weighted-backprojection method (right) and the developed ETtention framework (left). In the lower right of each images, a root-mean-square error is given, which is a measure of the fidelity of the reconstruction (the lower, the better). The contrast is markedly improved, which leads to better recognition of the intra-cellular organelles and macromolecular complexes, which is one of the key goals for NOTOX. It supports the choice of block-iterative methods as the basis of the reconstruction pipeline within which improved regularisations, noise models, and sampling strategies can be implemented to further improve the quality.

**Figure 4.49** A slice from 3D cryo-electron tomography reconstruction of a vitreous section of *S. cerevisiae* using weighted-backprojection (right) and our ETtention framework (left). Note the enhanced contrast in the enhanced reconstruction leading to improved recognition of the intra-cellular structures. RMS=root mean square.

To facilitate a quick adoption into a daily workflow and efficient data sharing and exchange both within the NOTOX consortium and later with partners from the SEURAT-1 Research Initiative in general, a communication layer between the ETtention framework and well-known community-standard tool IMOD has been implemented, together with a support for an industry-standard MRC data format.
4.6.5 Integrative and Predictive Computational Systems Biology in Toxicology

State of the Art

Mathematical models will be used to integrate information obtained from the iterative cycles of model predictions and experimental validations by *in vitro* experiments to eventually predict possible toxicity of the test compounds *in vivo*. For this purpose, histological human data can be used to provide the *in vivo* tissue architecture information and thereby complement the information obtained from the *in vitro* studies. Experimental data, modelling results as well as databank and literature data will eventually be combined in large-scale bioinformatics systems, extracting knowledge concerning long term toxicity and its prediction. Modern computational tools that allow systems to be pursued and studied are in abundance. There are now pathway analysis tools and datasets available from several companies (e.g. Ingenuity Systems, Cambridge Cell Networks, Genego) and academic sites (e.g. Reactome, KEGG, Biocarta, Cytoscape). The fact that these cover a wide range of processes and often contain genes and proteins in addition to chemicals, permits researchers from different fields to better communicate with each other. There are also a number of software packages that allow systems to be modelled and thus predictions to be made. The E-cell and Silicon Cell projects, for example, are international consortia attempting to create usable models of biological processes: such models have been met with some success in certain applications (e.g. Nakayama et al., 2005; Hornberg et al., 2007). However, these applications can require a more complete set of parameters than is often available for many systems. Missing data is not always a critical issue as it is increasingly possible to use simpler networks directly to make predictions. Most often, these predictions are of biomolecular interactions that have not yet been reported (e.g. Linding et al., 2007), but methods are emerging that use networks directly to predict macroscopic phenomena, such as toxic endpoints (e.g. ToxWiz).

Approach

Modelling of metabolic and regulatory networks: The NOTOX partner ‘Insilico Biotechnology’ focuses on model-based, quantitative prediction of hepatic metabolic and regulatory mechanisms related to acute overdose or repeated dose-effects of xenobiotics. In the first project year, particular focus was kept on the modelling of acute toxicity caused by overdoses of xenobiotics. Acetaminophen (APAP), which has also been recommended as a gold compound by ToxBank (see chapter 4.7), was chosen as model compound. APAP overdose leads to severe toxic side effects and even acute liver injury. The first steps to acute toxicity include (i) CYP mediated oxidation of APAP to the metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) (ii) depletion of cytosolic glutathione (iii) binding of NAPQI to mitochondrial membrane and (iv) excessive synthesis of reactive oxygen (ROS) and nitrogen species (RNS). The
further progress of toxicity comprises the mitochondrial damage due to ROS and RNS and downstream events leading to cell necrosis and apoptosis.

Spatial-temporal modelling of tissue toxicity and functional consequences: Recently, members of the NOTOX consortium have shown that tissue toxicity and regeneration can be simulated in spatial-temporal mathematical models (Hoehme et al., 2010). These models are based on tissue reconstruction from confocal laser scans and experimentally determined process parameters, which initially have to be obtained from the in vivo situation. One of the current open questions addressed in NOTOX is whether such computer models can be also used to predict long-term toxic effects.

Results

Modelling of metabolic and regulatory networks: Our model for short-term toxicity comprises APAP degradation, glutathione metabolism, and ROS/RNS synthesis (Figure 4.50) for the precise prediction of ROS appearance as an indicator of acute toxicity.

Figure 4.50 Model of acetaminophen (APAP) metabolism, glutathione metabolism and ROS- and RNS-synthesis in hepatocytes. APAP is degraded by phase II conjugation enzymes, UGTs and SULTs and by phase I CYP catalyzed oxidation to NAPQI. NAPQI is detoxified by GST-enzymes to NAPQI-GS. Therefore, glutathione has to be regenerated from the amino acids glutamate, cysteine, and glycine. NAPQI stimulates NO-synthesis and binds to the mitochondrial membrane causing oxidative stress resulting in an elevated synthesis of reactive oxygen species, O₂· and H₂O₂ and reactive nitrogen species, ONOO⁻.
The model has been pre-parameterised by literature values of kinetic parameters and compound concentrations and first simulations have been performed to test the model functionality. Moreover, the implementation of a comprehensive set of stratified or individualised enzyme concentration data, e.g. for CYP450 enzymes, will permit the estimation of corresponding concentration profiles and maximum non-toxic plasma levels.

Currently, a comprehensive dynamic hepatic model which displays important pathways of central carbon metabolism in the liver in absorptive state is being constructed by NOTOX partner ‘Insilico Biotechnology’. This model will further be extended by corresponding reaction steps and pathways which are active in a starving state in the liver. The switch between absorptive and starving states in the liver is strictly controlled by blood levels of specific hormones, most prominently, insulin and glucagon. Therefore, a first draft of a regulatory model which includes the insulin- und glucagon-induced signal transduction, as well as the resulting enzyme activity modification and gene regulation, has been constructed by ‘Insilico Biotechnology’. Signal transmission capability was successfully qualitatively adapted. Model validation will be conducted starting with the $^{13}$C-metabolic flux analysis in cooperation with the project partners.

Spatial-temporal modelling of tissue toxicity and functional consequences: It is well established that the hepatotoxic model compound CCl$_4$ causes a characteristic pattern of pericentral liver damage (Hoehme et al., 2010). From previous work, it is also known that detoxification of ammonia takes place in two compartments of the liver lobule (Schliess et al., 2012; Figure 4.51).

**Figure 4.51** Structural and functional organisation of ammonia detoxification along the liver lobule. The scheme depicts intercellular glutamine cycling between periportal and pericentral hepatocytes. Periportal glutaminase (GLNase) and pericentral glutamine synthetase (GS) are simultaneously active, resulting in a periportal breakdown and pericentral re-synthesis of glutamine. The extent of the GLNase-catalyzed ammonia (as ammonium cation, NH$_4^+$)
amplification determines the flux through the carbamoyl phosphate synthetase (CPS) that is a low affinity but high-capacity system for $\text{NH}_4^+$ detoxification. Glutamine consumed by the periportal GLNase is resynthesised in pericentral hepatocytes by the GS-catalyzed reaction from the $\text{NH}_4^+$ that escaped upstream urea synthesis (for details see Schliess et al., 2012).

Based on this knowledge, a metabolic model was established that considers compartmentalisation of ammonia detoxification. A system of ordinary differential equations representing the rate limiting reactions involved in the periportal (high capacity, low affinity) and the pericentral (low capacity, high affinity) ammonia detoxification was established. The parameters of the compartment model in the non-damage situation were calibrated by using an image processing and analysis chain as well as enzymatic measurements. By coupling the compartment model and the spatial-temporal liver model, it was possible to predict the impact of an in vivo intoxication induced by CCl$_4$ damage on the lobules (Figure 4.52). The model simulations are in a good agreement with experimentally determined data. In conclusion, it was shown that it is possible to use a tissue destruction and regeneration pattern to calculate its impact on liver metabolism. In the future, this approach will be applied to predict more complex scenarios and extend the model to long-term toxic effects. Moreover, the detoxification reactions shall be integrated into each individual cell.

Figure 4.52 The integrated model shows the spatial-temporal architecture of seven liver lobules together with the corresponding predicted concentrations of ammonia ($\text{NH}_4^+$), urea and glutamine. The tissue architecture was constructed based on an extension of the model in Hoehme et al. (2010). The plot shows the time-dependent changes in the concentration of ammonia, urea and glutamine during the induction of tissue damage by CCl$_4$ and regeneration as calculated by the metabolic model. The simulated tissue structure is from day 4 after the intoxication by CCl$_4$. 
4.6.6 Data Handling and Management: NOTOX Wiki Platform

The NOTOX consortium strongly focused on defining detailed experimental strategies and useful data handling in the first project year. Thereby, modelling groups and experimentalists intensively joined their expertise in designing experiments, models and data analysis. The NOTOX partner ‘Cambridge Cell Networks’ has established a web-based Wiki platform for communication of scientific information and data sharing between partners. For the intended integration of experimental data from various sources, it is essential to carefully design experiments in a well-documented and transparent way. Data acquired in these experiments will be stored in well-defined formats, allowing incorporation of data into the database in a retrievable manner. Processed and characterised ‘-omics’ data can, therefore, be superimposed onto maps of biological pathways in order to create and validate the hypothesised models of mechanism of action and toxicity of the reference compounds.

The first prototype for the content of the Wiki was established (Figure 4.53) based on the experience from an FP7 Project SYSCILIA. The first draft of a Wiki proposal was presented in the kick-off meeting in Saarbruecken in January 2011 and the feedback from all partners was integrated in the functional version of the Wiki. The NOTOX-Wiki content is continually refined with selected partners.

![Figure 4.53 The main page of NOTOX Wiki portal. Important links on this page are to the project webpage, other SEURAT projects, protocols for data collection, compound selection and ToxWiz database (a knowledge base from CCN).](image-url)
Furthermore, experimental protocols were discussed in NOTOX meetings and the efforts for harmonisation are ongoing. These comprise origin and pre-treatment of biological material, culture conditions, media, sampling and quenching, sampling frequency, sample preparation for final measurement, measurement methods and primary data handling. The NOTOX Wiki system is essentially supporting the documentation of these activities. A list of endpoints was discussed in the NOTOX meetings and is given on the NOTOX Wiki page. This list is continuously updated and is also in collaboration with other projects of the SEURAT-1 Research Initiative. The protocol describes endpoint data such as the use of dose-response curves for the EC50 estimation.

The established NOTOX Wiki page is central to sharing scientific information between project partners, especially for the exchange of huge data files. It enables better communication and synchronisation of overall experimental activities that are key to fulfilling the tasks and reaching the goals of the NOTOX project.

4.6.7 **Innovation**

The innovative focuses of NOTOX are the following:

- Establish new systems biology platform for long-term toxicity prediction with closely linked experimental and computational technologies comprising physiological and structural data.

- Apply organotypic cultures for long-term experiments preferentially using hESC derived cell lines.

- Study and model physiological status and dynamics using various ‘-omics’ technologies, i.e. epigenomics, transcriptomics, proteomics, metabolomics and fluxomics.

- Determine structural toxic effects using 3D cryo-electron tomography and optical and confocal microscopy methods and model such effects for the prediction of human toxicity.

- Create large multi-scale computational models supported by databases for the prediction of human long-term toxicity from in vitro data.

**Innovative strategies in the first phase of the project**

**Guided cell and tissue modelling for organotypic cultures:** The NOTOX project aims at establishing and applying organotypic cultures for long-term repeated dose toxicity prediction. It is, therefore, also very important to characterise such organotypic cultures and demonstrate their suitability in such studies. The ongoing work in NOTOX is focusing on the dynamics of
organotypic tissue formations (Figure 4.54). In this regard, the experimental data generated within NOTOX is used for modelling. The established models will be fitted and optimised with the biochemical data obtained.

Figure 4.54 In-vitro 3D HepaRG culture (top) and in-silico (bottom). Individual-based models can help to identify the correct mechanisms on the cellular and molecular scale, leading to the phenomena on the tissue scale. The simulations suggest that a kind of long-range interaction between cells is necessary to guide the cells migration to explain the aggregation process observed in the droplets, if the number of cells that were originally seeded is large (top row). Gravity and cell-cell-adhesion (bottom row) alone do not seem sufficient to guarantee the formation of an aggregate at the experimentally observed time scales for large cell population sizes.

Another aspect of structural characterisation is the 3D tomography reconstruction. In this regard, the methods being developed focus on fully 3D block-iterative ones, with the emphasis on the efficient parallel implementation on both CPUs and GPUs and the support for advanced a priori regularisations, noise models, and improved sampling.

Large multi-scale models for toxicity: For long-term repeated dose toxicity, modelling efforts are required at various levels (Figure 4.55). These multi-scale models can improve the predictivity of a system. The assessment of stratified or individualised risk of a drug by implementation of genome-related data, e.g. enzyme expression and activity, into model-based predictions, would be of great value. Drug-induced epigenetic alterations as well as interindividual differences in the hepatic epigenome, in relation to the alteration in the transcriptome and capacity for drug metabolism and drug toxicity, will be studied and modelled within the NOTOX project. Model-based predictions of long-term repeated dose effects on the basis of combined cellular, endobiotic and xenobiotic related, metabolic and regulatory network systems, will be carried out.
Figure 4.55 Replacement of cost- and time-intensive preclinical studies by fast computational approaches. Detailed in silico hepatocyte models which are validated by experiments, permit the prediction of dose-dependent concentration-time profiles of xenobiotics. The knowledge of appropriate toxicity thresholds and the implementation of population data into predictive models enables an individualised or stratified estimation of maximum serum concentrations of drugs and drug-metabolites, as well as an individualised risk assessment.

Use of high-performance computing systems for model verification, validation and prediction of large-scale molecular networks (1,000+ compounds) is possible with the help of NOTOX partners (INSIL).

4.6.8 Cross-cluster Cooperation

Three of NOTOX partners are also participating in other cluster projects, namely: DETECTIVE, SCR&Tox and COSMOS. The exchange of information and experiences within these projects will be of global benefit to these projects. Various collaborative efforts have been initiated between NOTOX and COSMOS. These are especially with ‘Mario Negri Institute’ for compound-binding studies and ‘Insilico Biotechnology’ for the in silico dynamic liver model.

In March 2011, NOTOX partner ‘Saarland University’ received ToxBank representatives to discuss various data to be acquired within NOTOX. NOTOX also welcomed the representatives of the ToxBank project to visit NOTOX partners for data management. Close collaboration with ToxBank on data management and compound selection was facilitated. NOTOX partner ‘Cambridge Cell Networks’ is helping ToxBank with practical issues of databases, data warehouse and data management.

Exchange of data with other projects of the SEURAT-1 Research Initiative will be made
primarily by using databases and data management structures supplied by NOTOX via its WiKi platform in collaboration with the data warehouse in ToxBank.

4.6.9 **Expected Progress within the Second Year**

In the second year, a progress meeting was already held in Amsterdam in January 2012. The NOTOX partners will carry out the experiments during this year using 2D and 3D HepaRG cultures for various ‘-omics’ techniques. Muti-scale joint pilot experiments are presently planned with intense discussions between the experimentalists and the modelers. Organotypic cultures will be further optimised for long-term repeated dose toxicity assessment. In this regard, serum-free cultivation of HepaRG cells is investigated to facilitate the proteomics studies, as well as to minimise epigenomic marks due to fetal calf serum.

The goals for the 2nd project year of NOTOX are:

- To establish serum free-cultivation of HepaRG cells in 2D and 3D cultures.
- To further investigate culture conditions of the spheroids, e.g. at different oxygen concentrations, including modelling of nutrient and oxygen diffusion within the spheroids and investigation of initial seeding number and impact on spheroid size.
- To carry out physiological and metabolic characterisation of organotypic cultures.
- To make long-term maintenance (two and more weeks) of HepaRG cells with optimised conditions and baseline characterisation for the various ‘-omics’ techniques available.
- To perform repeated dose testing of acetaminophen and other selected compounds using established cultivation methods (3D spheroids, sandwich culture and bioreactors).
- To design and execute a joint large-scale pilot experiment by various groups of NOTOX. This investigation focuses on the short-term toxicity of acetaminophen, including acetaminophen metabolism, glutathione depletion, and ROS synthesis. Metabolomics, fluxomics, proteomics, epigenomics and transcriptomics analyses are also included.
- Conduction of experiments on HepaRG cultures, including sample preparation and metabolite measurements, proteome measurements, possibly also transcriptome analysis and model verification of kinetic parameters.
- To improve MS-based proteomics studies by means of a new proteomics data analysis module, which will allow interpreting quantitative label-free (e.g. spectral counting) and label-based (e.g. LC-SRM) MS data. Such a module is
Currently being developed and will soon be added to MSDA.

- To extend the signalling model by drug induced signalling pathways.
- To implement stratified and individualised enzyme data from NOTOX partner ‘Karolinska Institutet’ data bank.
- To simulate individualised acetaminophen metabolism and short-term toxicity.
- To improve the contrast, resolution and throughput of 3D cryo-electron tomography reconstructions through efficient novel regularisation schemes, noise models, and improved sampling strategies.
- To optimise the reconstruction of macromolecular complexes of interest focusing on sub-tomogram averaging. This technique allows orienting, aligning and merging 3D structures within the tomograms, and, thus, increasing the signal-to-noise ratio.
- In order to maintain a straightforward integration of data into the databases, easy accessibility and flexibility for the intended data cross-integration with other projects of the SEURAT-1 Research Initiative NOTOX partner ‘Cambridge Cell Networks’ will design data handling prior to actual data collection, following a strict systems biology protocols within the NOTOX project. The designed protocol for time-point and end-point data collection will enable the experimentalists to collect and communicate data in a well-structured, fully-documented way that is necessary in a systems biology approach. This will enable integration and analysis of complex data from multiple experimental sources required by modelling groups.
- The acetaminophen model validation will be carried out within the 2nd year, in close cooperation with the NOTOX partners conducting acetaminophen acute toxicity experiments on HepaRG monolayer cultures. The developed models capable of simulating long-term, repeated-dose drug effects will be integrated into larger models combining metabolic and regulatory pathways in the second half of 2012. This enables the combined simulation of xenobiotic and central hepatic metabolism considering detoxification pathways, xenobiotics-induced signalling pathways, enzyme modifications, gene regulation and, thus, the induced changes in hepatic metabolism.

4.6.10 Future Perspectives

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

In the NOTOX project, eventually easily applicable methods of analyses will be developed, so that they can be readily transferred to other cellular systems, e.g. those developed/optimised in other projects of the SEURAT-1 Research Initiative. In vitro test systems are of utmost importance for animal-free toxicity assessments. In NOTOX, we have already made significant progress in the establishment of long-term 3D organotypic cultivation techniques that are considered a major part of long-term toxicity assessment systems. The ultimate goal is to create cellular systems, which are as simple as possible, e.g. using sandwich culture or spheroid cultivation using new techniques providing a high degree of reproducibility and predictivity. Miniaturised cultures, e.g. single spheroids or even functional organoids, that are presently limited in their applicability due to the lack of sufficiently sensitive analytical techniques, will gain increasing relevance also for a systems-wide characterization.

Future in vitro long-term toxicity testing system will comprise predictive in vitro cellular systems most likely based on stem cell technology that is expected to deliver tissue-specific cells. These seem very promising for long-term cultivation. They will even be assembled in a way to simulate the behaviour of a whole organism to a large extent, e.g. as body-on-a-chip. An alternative approach would be the establishment of cells with predictive reporter constructs.

Multi-scale mathematical and informatics computer models will describe the mechanistic events from molecular to tissue to organism levels, thus improving the predictive power. Cellular and molecular ‘signatures’ will be identified, allowing the prediction of long-term toxicity using ESC derived cells and using different organs. This can be achieved by extensive collaboration within the SEURAT-1 Research Initiative. For a systems biology approach, this will essentially provide an excellent starting point to further refine strategies for obtaining improved predictivity 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 characterization of the toxicological consequences. Ultimately in the future, the goal is a routine assessment and semi-automatic reasoning about general compounds and toxicological mechanisms. This will require the study of significantly smaller complexes and more subtle structural changes, to recognise the adverse effects as early as possible. Finally, the multi-scale models should allow in vivo extrapolation of long-term toxicity prediction in humans, which will be a great advance in the direction of alternatives to animal testing.
References


Recent key references of project members (last 3 years) related to the field of research


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

Emilio Benfenati, Glenn Myatt, Jeffrey Wiseman, Barry Hardy

4.7.1 Introduction and Objectives

ToxBank (www.toxbank.net) establishes a dedicated web-based warehouse for toxicity data management and modelling, a ‘gold standards’ compound database and repository of selected test compounds, and a reference resource for cells, cell lines and tissues of relevance for in vitro systemic toxicity research carried out across the SEURAT-1 Research Initiative. The project develops infrastructure and service functions to create a sustainable predictive toxicology support resource going beyond the lifetime of the Research Initiative.

In this project report we will focus on the two main activities addressed during the first year within ToxBank: the identification of reference compounds (‘gold compounds’) for toxicity testing and the development of the ToxBank Data Warehouse.

These activities have involved all other projects of the SEURAT-1 Research Initiative in a choral effort. The identification of the reference compounds required a deep discussion within SEURAT-1 aimed to achieve not only a list of chemicals, but most important, a common aim about the harmonised way to explore the toxicity processes we want to address. Each reference compound has been discussed regarding its contribution it would bring to the overall strategy of the SEURAT-1 Research Initiative, and also the possible limitation which could affect its use. As a result, each reference compound is annotated with a broad series of characteristics, driving the planning of the experiments, its use, and the successive interpretation of the results. The collection of data, structures and properties, represents a wide set of compounds, and we will take advantage of such a scheme to increase the number of reference compounds later on when needs of further chemicals may appear.

The second activity we will present refers to the ToxBank Data Warehouse. Also, these activities required a broad consultation of all projects, in order to understand their needs in terms of data and information, what kind of results they obtain, how the data are processed,

1 - On Behalf of the ToxBank consortium
and what use is anticipated of the results. The ToxBank Data Warehouse will represent the memory of SEURAT-1 Research Initiative, which will be a living system, able to retrieve and process the information.

These activities are related to the following objectives of ToxBank (a complete overview about all ToxBank objectives are given in Hardy et al. (2011)):

➤ 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

➤ Select ‘gold standards’ test compounds (‘Gold Compounds’) having high-quality data and providing chemical and biological diversity across a range of repeated-dose toxicity endpoints

➤ Create a ToxBank Gold Compound Database for the import, curation, acceptance and storage of quality data related to Gold Compounds

➤ Establish a Data Warehouse of Linked Resources which house and provide access to a centralized 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 to the TBDW

➤ 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.)

4.7.2 Mechanism-based Selection of Reference Compounds for Toxicity Testing Procedures

Rationale

The selection of standard reference compounds is a critical issue in any research programme
that involves many research groups from different scientific disciplines and needs to be done according to the overarching goals or strategy of the program. In case of the SEURAT-1 Research Initiative, the strategy and goals were outlined in the first Annual Report (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’.

The following core concepts from the SEURAT goals govern the compound selection strategy:

1. **Chemical space.** The SEURAT strategy encompasses ‘any substance’. Thus, the initiative must cover a breadth of chemical classes that include cosmetic ingredients, agricultural and industrial chemicals, and pollutants, among others. At the same time, our understanding of human toxicity arises primarily from clinical observations within the chemical space of marketed drugs. We are challenged, therefore, to select standards that facilitate extrapolation from well-studied drug toxicities to this broader space.

2. **Promiscuity.** Promiscuity refers to lack of structural specificity in ligand binding. The concept originated with high throughput screening and has been extended to the characterization of toxicants (Shoichet, 2006; Azzaoui et al., 2007). It derives from the observation that small, hydrophobic ligands tend to have binding affinity for multiple different proteins, and conversely, receptors with large hydrophobic pockets tend to bind multiple diverse ligand structures. Promiscuity is clearly relevant to a strategy that must span a broad chemical space and was explicitly considered in selection of reference compounds for SEURAT-1.

3. **Mode of Action (MoA).** The underlying assumption of the SEURAT strategy is that we can identify MoAs that are demonstrably relevant to human toxicity based on the demonstrated adverse events of marketed drugs in humans. These MoAs then become intrinsic risk factors for toxicity that are independent of chemical space.

It is important to state that, while the overarching SEURAT goal is to assess human safety, the goal of this compound selection strategy is narrower – i.e. the establishment of MoA-based in vitro assays. Thus, while drugs are necessary for identifying relevant MoAs, a drug may affect a complex array of biological pathways; and non-drug compounds may be preferable for characterizing a single MoA. Use of non-drug standards when appropriate can have the additional benefit of demonstrating the relevance of the SEURAT program across multiple chemical classes. Other issues such as prediction of exposure and ADMET properties, although critical to predicting human toxicity, were not a determining factor for compound selection _per se._
A limitation of an MoA-based strategy is that our understanding of MoA for even the best known toxicants is incomplete. The challenge is to select reference standards despite our incomplete understanding, and the opportunity is to select compounds that at the same time will enable us to increase our understanding of MoA.

4. Repeated dose toxicity. While in many cases the biological rationale behind repeated dose toxicity is not fully understood, in the context of the MoA for toxicity, there are only two possibilities. Either the MoA leading to repeated dose toxicity is the same as that for acute toxicity or it is different. To illustrate, carbon tetrachloride at high doses causes acute wide-spread hepatic, but the liver recovers from this insult via tissue regeneration. In contrast, low repeated doses cause a more limited and localized necrosis, but in this case the tissue response is fibrosis rather than regeneration (Hoehme et al., 2010). This is an example where the primary MoA is the same for both acute and repeated dose toxicity, i.e. necrosis of hepatocytes. The repeated dose toxicity of phenobarbital, in contrast, is proposed to result from changes in locus-specific DNA methylation patterns, an MoA distinct from acute biological responses (Schwarz & Mahony, 2011).

5. The compound selection strategy must obviously be based on an understanding of MoAs that underlie repeated dose toxicity so that these MoAs are adequately represented in the in vitro assays. The difficulty behind this simple statement is again illustrated by carbon tetrachloride. Many compounds cause hepatic cell death, but not all of these cause fibrosis upon repeated low exposures. The challenge is to understand MoA at a level of detail sufficient to distinguish these compounds.

The SEURAT-1 Research Initiative is addressing hepatic, cardiac, renal, neuronal, muscle, and skin toxicities. The compound selection strategy to date has been developed only for hepatic and cardiac toxicities, and will be expanded with time to other tissues. By agreement across the cluster, carcinogenicity and mutagenicity are excluded from consideration since they are being addressed elsewhere (Vinken et al., 2008).

General Selection Criteria

Reference compounds are selected primarily based on their relevance to MoA in human toxicity. However, additional criteria apply generically to all compounds to ensure their applicability for cell-based in vitro assays. These are listed in Table 4.5. Reference ‘-omics’ profiles from the literature are important to this project since ‘-omics’ profiling will be used to characterize cellular responses to toxicants, and comparison to previously observed profiles is one strategy for validating cellular assay systems. Criteria for acceptable physical properties were established to ensure ease of handling in in vitro assays (Knudsen et al., 2011).
Table 4.5 Generic criteria for selecting reference compounds

<table>
<thead>
<tr>
<th>Define, confirmed structure and isomeric form</th>
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<tr>
<td>Stable to storage, light, freeze thaw</td>
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<tr>
<td>Soluble in buffer at 30 times the in vitro IC50 for toxicity*</td>
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<tr>
<td>Solubility in DMSO at 100x buffer solubility</td>
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<tr>
<td>Insignificant binding to plasticware**</td>
</tr>
<tr>
<td>Available commercially at &gt;95% purity (&gt;99% preferred)</td>
</tr>
<tr>
<td>Non-volatile</td>
</tr>
<tr>
<td>Gene expression, proteomics, metabonomics/fluxomics, and/or epigenomics profiles known †</td>
</tr>
<tr>
<td>Bioactivated (hepatotoxins) ††</td>
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</tbody>
</table>

* Sparingly soluble compounds may be assayed for solubility in serum and the minimum percent serum to ensure solubility in vitro specified.

** This property will be measured when a sample of compound becomes available.

† Literature data for at least one, but not necessarily all, of the ‘-omics’ data types is desired. This requirement can be waived in special cases.

†† This is a desirable but not required property of hepatotoxins.

Compounds that violate one or more of the selection criteria have been accepted by agreement across the gold compound working group if desirable properties override negative ones (e.g. CCl₄ is volatile and poorly soluble but extensively characterized for its pro-fibrotic activity).

The initial compound selection strategy focused on marketed drugs as the sole source of reference compounds, and we agreed to reject compounds if toxicity in humans was idiosyncratic. Since adverse events have been labelled as idiosyncratic based on frequencies of observation ranging from 1:10 to more than 1:10,000 in the literature, however, this criterion did not provide an objective threshold for accepting or rejecting a compound, and the criterion was dropped. In addition, we have proposed compounds that are not marketed drugs in cases where they will aid in establishing assays based on well-defined MoAs.

Reactive Compounds

Reactive molecules, i.e. compounds with alkylating and redox activity, feature prominently in the MoA of the hepatotoxic drugs (Gómez-Lechón et al., 2010), and the same appears to be true in the chemical space of cosmetic ingredients (Vinken et al., 2011). In the context of compound selection principles outlined above, chemical reactivity represents an example...
of ligand promiscuity. Among these reactive molecules, quinones are a prototypical reactive moiety so we will start our discussion with this class of compounds, and specifically with the archetypical toxicant, acetaminophen (Tang, 2007; Vinken et al., 2011).

**Acetaminophen:** Quinones are redox active and are differentiated by their reduction potential, which varies by more than 1 V (>15 logs in reactivity), and by whether they are also alkylating agents (Song & Buettner, 2010). Highly electrophilic quinones with high reduction potential are exemplified by acetaminophen, which is the reduced, hydroquinone form of N-acetyl-p-benzoquinone imine (NAPQI). Since quinones in this class are strong oxidizing agents, the reduced form is stable and oxidation to the cytotoxic quinone form will require a strongly oxidizing system such as cytochrome P450, which means that toxicity for this class of compounds will be observed primarily in the liver and kidneys.

Acetaminophen is generally considered a very safe drug on repeated low dose exposure but can progress rapidly to liver failure at exposures above a safe threshold. This is consistent with classic models of tissue repair in toxicant-induced tissue injury in which injury progresses to organ failure when the capacity for repair is exceeded (Mehendale, 2005). A fundamental question for prediction of repeated dose toxicity is why this compound does not show fibrosis at low doses, in contrast to toxicants such as CCl₄, allyl alcohol, and methotrexate.

Many drug-derived toxicants such as acetaminophen have multiple reactivities and affect multiple biological pathways, so that understanding MoA at the molecular level can be problematic. NAPQI is an alkylating thiol reagent and an oxidizing agent that oxidizes thiols as well as blocking electron transport via inhibition of complexes I and II (Burcham & Harman, 1991; Martin & McLean, 1995; Dietze et al., 1997; Chen et al., 1999; Chen et al., 2009; Hinson et al., 2010). In order to begin differentiating these MoAs, we propose iodoacetamide (a thiol alkylating agent) and rotenone (an inhibitor of complex I) as reference standards. Understanding the dominant MoA may have major implications in comparing the toxicity of acetaminophen to that of repeated dose toxicants such as doxorubicin and allyl alcohol in the discussions following.

**Doxorubicin:** Doxorubicin is a quinone with low reduction potential (Song & Buettner, 2010) that shows repeated dose cardiotoxicity. The stable species of quinones in this class is the oxidized form, so that activation by strongly oxidizing P450 systems is not required, and the compounds are toxic to many cell types. The major chemical reactivity of doxorubicin is redox cycling, by which cellular flavoproteins catalyze reduction of the quinone by NAD(P)H and the reduced hydroquinone is re-oxidized by oxygen, which may also be enzyme catalyzed. The most important result is to interfere with cellular redox processes such as the electron transport chain (Pointon et al., 2010), while to the extent that free radicals are generated in
the process, oxidative damage to proteins and DNA can also occur. Similar quinones such as menadione are also arylating thiol reagents (Henry & Wallace, 1995), but in the chemical space of the anti-tumor antibiotics, the sites of arylation are blocked, and only redox cycling is observed.

Doxorubicin also intercalates into DNA and causes oxidative DNA damage, which has been an historical focus for the anti-tumor toxicity of this class of compounds (Lüpertz et al., 2010). The central question for doxorubicin, therefore, is whether the MoA for repeated dose toxicity is due to cytotoxicity via redox cycling that accumulates over time or is due to DNA damage that manifests itself slowly in an organ that has low levels of cellular replication. DMNQ, which has comparable redox activity but low affinity for DNA, is proposed as a reference compound for elucidating the dominant MoA of cardiotoxicity (Pointon et al., 2010).

Characterization of the full class of low potential quinones must address alkylating activity, for which we are proposing iodoacetamide as MoA reference standard.

**Allyl alcohol:** Allyl alcohol is a reference standard for liver fibrosis (Jung et al., 2000; Mehendale, 2005). It is oxidized to acrolein by alcohol dehydrogenase, which is localized to the liver. Acrolein is a potent thiol alkylating agent that, for comparison, is 1000-fold more reactive than iodoacetamide (Tacka et al., 2002). Although the major reactivity is alkylation, acrolein is also a radical chain propagator so that free radical reactions are also possible. Acetaminophen and carbon tetrachloride cause necrosis in the centrilobular region, where P450 activity is high (Oinonen & Lindros, 1998), while allyl alcohol causes necrosis in the periportal region (Yin et al., 1999). As discussed above for acetaminophen, a question to address for repeated dose toxicity is why allyl alcohol, methotrexate, and CCl₄ all cause fibrosis, despite showing activity in different regions of the liver and different MoAs for toxicity at the molecular level, while acetaminophen does not.

**Carbon tetrachloride:** CCl₄ is a reference standard for liver fibrosis. The chemistry of CCl₄ is inherently different from toxicants considered above because the primary reactive species is a free radical, trichloromethyl radical. Trichloromethyl radical is a very high energy species that is formed by reduction and is accessible via the cytochrome P450 (primarily 2E1) system. Toxicity is observed primarily in the liver and can arise via direct trichloromethyl adduct formation or by oxidation of unsaturated fatty acids to Δ, β-unsaturated aldehydes, which are themselves alkylating agents (Weber et al., 2003). In addition to generation of reactive metabolites, extensive lipid oxidation can itself disrupt membranes.

The central question for this reference standard is whether or not this different chemistry represents a different MoA in terms of the biological processes targeted, especially when compared to the alkylating agents. For example, although toxicity is observed in perivenous
rather than in periportal hepatocytes (Oinonen & Lindros, 1998; Yin et al., 1999) the chemistry of reactive aldehydes generated from lipids by CCl₄ is similar to that of acrolein generated from allyl alcohol. Perhaps for this reason, gene expression patterns induced by CCl₄ cluster with those for allyl alcohol when multiple hepatotoxins are compared in rat liver (Waring et al., 2001).

**Aflatoxin B1:** Aflatoxin B1 is representative of epoxide reactive centers and is of interest because it induces apoptosis. Although well known as a hepatic carcinogen, aflatoxin B1 induces apoptosis at higher doses, which is the toxicity of interest. The DNA-alkylating epoxide, which is formed by oxidation in the liver, has 3-4-fold preference for mitochondrial DNA over nuclear DNA. Because repair of mDNA is also slower, there is a preferential reduction in mitochondrial protein synthesis compared to cytosolic synthesis, and one source of toxicity may be interference with mitochondrial protein synthesis (Niranjan et al., 1982).

However, the epoxide has a very short half-life and is hydrolyzed to a dialdehyde, which is considered the primary species leading to apoptosis. The dialdehyde is a lysine reagent and therefore will target different proteins than the more common thiol-targeting reactive groups (Guengerich et al., 2002). Aflatoxin B1 induces cell death in cultured cells via apoptosis without depletion of ATP, which is different from the effect of thiol reagents and implies different protein targets (O’Brien et al., 2000). This reference compound, therefore, provides an opportunity to profile a much different chemically reactive center. In addition, the extent to which alkylation of mDNA is a factor in short term toxicity is not clear and is a question that may be addressed in the course of assay characterization.

**Chlorpromazine:** The repeated dose toxicity of interest for chlorpromazine is cholestasis (Mohi-ud-din & Lewis, 2004). Although the compound can generate an immunologic reaction, there is a dose-related component to cholestasis, suggesting that intrinsic toxicity is at least a contributing factor (Moradpour et al., 1999). Chlorpromazine is oxidized to the 7-hydroxy quinone imine in hepatocytes, which has reactivity similar to acetaminophen (Wena & Zhoub, 2009). However, the parent compound is itself toxic and can be oxidized by peroxidases to a free radical that might more resemble CCl₄ in its MoA (Eghbal et al., 2004).

The parent binds to phospholipid bilayers, which is the source of the pharmacological activity for this drug (Seeman, 1977; Anderson & Borlak, 2006) and is a relatively potent ATP synthase inhibitor (Nadanaciva et al., 2007). As will be discussed further below with respect to amiodarone and tamoxifen, these results together imply a role for membrane disruption in cytotoxicity, specifically in the inhibition of oxidative phosphorylation.

The compound diffuses freely across membranes and has an affinity for bile salts so that it may concentrate in the bile ducts. The key question for this compound is whether its
cholestatic activity can be predicted by an in vitro cell culture system. Additionally, given multiple proposed MoAs for toxicity, it is a question whether a single dominant MoA can be resolved by comparison to other standards.

Iodacetamide: Iodoacetamide is proposed as an MoA standard that alkylates GSH and protein thiols. Since iodoacetamide does not require metabolic activation, it can be used to characterize the effects of a thiol reagent across multiple cell types with a reagent that is itself devoid of any complicating redox activity.

Iodoacetamide was used in early studies that unravelled the pathways of energy metabolism (Dickens, 1933). Thus, while many modern discussions of reactive molecules focus on mitochondrial disruption and free radical generation (Gómez-Lechón et al., 2010), one of the values of this reagent is a historical literature on blocking energy production via inhibition of glycolysis in the cytosol. While iodoacetamide depletes GSH, this in itself is not a cause of cytotoxicity (Redegeld et al., 1992; Schmidt & Dringen, 2009). This is because depletion of GSH by alkylation has minimal effect on the GSH/GSSG ratio and therefore the redox potential of the cell. Instead, protein active sites can be 100- to 1000-fold more reactive than GSH, and these proteins are the targets that are most sensitive to thiol reagents. Glyceraldehyde phosphate dehydrogenase is one of the most reactive of these proteins, and iodoacetamide-induced cytotoxicity is attributed to inhibition of glycolysis and depletion of ATP resulting from alkylation of this enzyme. This reactivity is intrinsic to the protein and not the inhibitor, so that acetaminophen also alkylates glyceraldehyde phosphate dehydrogenase (Dietze et al., 1997). The fact that cells are most sensitive to iodoacetamide when glucose is the energy source and less sensitive when pyruvate or amino acids are supplied as energy source (Allen et al., 2005), provides an added dimension to our understanding of MoAs based on thiol depletion.

However, signalling pathways initiated by depletion of GSH or targets of alkylation other than glyceraldehyde phosphate dehydrogenase may determine the tissue responses to chronic long-term exposure to this reagent (Stevens et al., 2000). Fluorescent tags derived from iodoacetamide are available to enable an analysis of the specific proteins targeted by this reagent (Dennehy et al., 2006; Shin et al., 2007; Wong & Liebler, 2008; Jacobs & Marnett, 2010). This reagent, therefore, is a tool that will help us to identify key points of interference for thiol-directed alkylating agents with respect to both cellular toxicity and communication of the cell with the surrounding tissue.

DMNQ: Dimethoxynaphthoquinone (DMNQ) was developed in order to evaluate the redox cycling MoA separate from the alkylating MoA of low potential quinones (Gant et al., 1988). DMNQ is a poor DNA intercalator and has also been proposed as a standard for studying redox cycling activity separate from the DNA-directed activities of anti-tumor quinones such as doxorubicin (Pointon et al., 2010).

Rather than inhibiting the electron transport chain as NAPQI does, DMNQ and related
quinones can substitute for ubiquinone and bypass complex I of the electron transport chain to transfer reducing equivalents from NAD(P)H directly to cytochrome c (Haefeli et al., 2011; Wen et al., 2011). Reduction of quinones in this case is accomplished by DT diaphorase in the cytosol (Floreani & Carpenedo, 1995; Karczewski et al., 1999).

Cytotoxicity is observed in a variety of cell types (except platelets, (Bresgen et al., 2003) and is associated with oxidation of GSH (e.g. hepatocytes, (Gant et al., 1988)). The presumed primary MoA is unregulated oxidation of NADH in the cytoplasm to drive down the cellular reduction potential. This reaction is catalytic if the reduced quinone is re-oxidized by oxygen and/or cycles with extracellular oxidized quinone by diffusion (Watanabe and Forman, 2003). The fact that DMNQ is not cytotoxic to platelets implies that cytotoxicity may be DNA-dependent (Wilmes et al., 2011).

**Promiscuous Ligands**

The concept of promiscuous ligands and receptors was presented at the beginning of this chapter (see the conceptual considerations above). In this section we consider promiscuous ligands, where promiscuity derives from hydrophobic interactions. We have designated two prototypical drug toxicants that fit into this category as reference standards, and we suggest that membrane disruption may be a significant MoA for known toxicants. Oligomycin and FCCP, which are considered under the heading of MoA Standards for Oxidative Phosphorylation, are related to this class.

**Valproic acid**: Valproic acid is a hydrophobic compound with very high exposure at clinical doses. It is selected as a reference compound for steatosis and cytotoxicity (RxAbbott, 2011). It is a fatty acid analogue that competes with fatty acids in the beta-oxidation pathway, which is the presumed cause of steatosis (Kesterson et al., 1984). It is also oxidized to an \( \alpha,\beta \)-unsaturated acid that is an alkylating reagent, but metabolism is not necessary for cytotoxicity (Kiang et al., 2011). The exact mechanism(s) of pharmacological activity and cytotoxicity are not yet known (Rosenberg, 2007). However, the drug has a broad range of known activities, including inhibition of histone deacetylases, increased GABA activity via multiple mechanisms, attenuation of the NMDA receptor, inhibition of Na\(^+\) channels, inhibition of voltage dependent L-type Ca\(^{2+}\) channels and inhibition of voltage-gated K\(^+\) channels (Phiel et al., 2001; Rosenberg, 2007; Chateauvieux et al., 2010). These multiple activities place this toxicant in the category of promiscuous ligands.

**Amiodarone**: Amiodarone induces steatosis, phospholipidosis, and cell death. This drug is a cationic amphiphile that induces phospholipidosis via the classical mechanism of binding to phospholipids (Anderson & Borlak, 2006). Amiodarone inhibits beta oxidation of fatty acids, which is inferred to be the cause of steatosis (Kaufmann et al., 2005). It also inhibits ATP synthase plus, more weakly, other steps in the electron transport chain (Nadanaciva et al.,
2007). Amiodarone positions in the hydrophobic core of the lipid bilayer where it alters lipid dynamics; and these interactions are believed to affect ion transport (the pharmacological effect of the drug) and oxidative phosphorylation (Anderson & Borlak, 2006). Thus the promiscuous activity of this drug is not necessarily a result of binding to multiple protein targets but results at least in part from lipid bilayer disruption. The observation of both binding to phospholipids and inhibition of ATP synthesis is reminiscent of the activity of chlorpromazine discussed above.

Promiscuous Receptors and Cellular Responses

In this section we suggest protein systems that are relevant to compound selection based on the demonstrated promiscuous response to toxicants.

hERG: Inclusion of the hERG ion channel as a target for reference compounds is based on its demonstrated promiscuous binding activity, which has been demonstrably problematic in the development of safe drugs (De Ponti et al., 2002). E4031 (N-[4-[1-[2-(6-Methylpyridin-2-yl)ethyl]piperidine-4-carbonyl]phenyl]methanesulfonamide) was synthesized to be a specific inhibitor of hERG ion channels and has been selected as the standard for this receptor (Miyake et al., 1990).

Redox indicators: Nuclear factors Nrf2 and Hif-1α are indicators of reductive and oxidative equivalents, respectively, available to the cell. They represent promiscuous responses in the sense that changes in the availability of redox equivalents are induced by a wide array of toxicants via a variety of molecular mechanisms (Table 4.6).

Table 4.6 Hit rates from TOXCAST transcription factor screens*.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Hit Rate</th>
<th>Biological Pathways of Interest**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nrf2</td>
<td>53%</td>
<td>response to oxidative stress</td>
</tr>
<tr>
<td>HIF1α</td>
<td>8%</td>
<td>hypoxia and angiogenesis</td>
</tr>
<tr>
<td>PXR</td>
<td>76%</td>
<td>induction of metabolizing enzymes</td>
</tr>
<tr>
<td>AHR</td>
<td>17%</td>
<td>induction of metabolizing enzymes</td>
</tr>
<tr>
<td>CAR</td>
<td>1%</td>
<td>induction of metabolizing enzymes</td>
</tr>
<tr>
<td>LXRA/ß</td>
<td>7%</td>
<td>lipid/cholesterol homeostasis</td>
</tr>
<tr>
<td>FXR</td>
<td>&lt;0.3%</td>
<td>bile acid homeostasis</td>
</tr>
<tr>
<td>RARα</td>
<td>16%</td>
<td>regulation of GSH</td>
</tr>
<tr>
<td>RXRα</td>
<td>&lt;0.3%</td>
<td>lipid/xenobiotic homeostasis/metabolism</td>
</tr>
<tr>
<td>PPARα</td>
<td>3%</td>
<td>lipid metabolism/glucose homeostasis</td>
</tr>
<tr>
<td>PPARγ</td>
<td>47%</td>
<td>lipid metabolism/glucose homeostasis</td>
</tr>
<tr>
<td>ERα</td>
<td>29%</td>
<td>endocrine</td>
</tr>
<tr>
<td>AR</td>
<td>&lt;0.3%</td>
<td>endocrine</td>
</tr>
</tbody>
</table>

* Data compiled from Martin et al., (2010)

** Pathway indicated a historical classification that oversimplify the roles of HHRs especially.
**Nrf2** is a transcription factor that binds to the antioxidant response element (ARE) and induces the transcription of a number of phase II and antioxidant enzymes. Amongst these are enzymes involved in glutathione synthesis (including; glutathione cysteine ligase (GCL), glutathione cysteine ligase modifier (GCLM), glutathione synthetase (GS) and glutathione reductase (GSR)). Under steady state conditions Nrf2 is bound to its cytosolic inhibitor Keap-1 and targeted for ubiquitination and subsequent proteolysis. Oxidation or alkylation of specific cysteine residues of Keap-1 liberate Nrf2 allowing it to evade ubiquitination and enter the nucleus to enact its diverse anti-oxidant protection. It is activated by thiol reagents, both alkylating and oxidizing; and of the compound standards proposed above, is activated by iodoacetamide, acrolein (from allyl alcohol), DMNQ, doxorubicin, and acetaminopen (Tirumalai et al., 2002; Copple et al., 2008; Pointon et al., 2010). Activation by CCl$_4$ is observed but is weaker (Randle et al., 2008). However, activation of Nrf2 has not been reported for chlorpromazine. If this negative result were confirmed in studies of the SEURAT-1 Research Initiative, it would indicate that the reactive 7-hydroxy quinone imine metabolite, which should resemble NAPQI in its MoA (Wena & Zhoub, 2009), does not contribute significantly to the toxicity of this compound – a useful contribution to understanding the MoA for chlorpromazine. On the other hand, it is unexpected that NRF-2 is activated by rotenone (Martin et al., 2010), since this reagent retains high cellular NADH levels and therefore maintains the cellular reduction potential. A possible explanation relevant to efforts of the SEURAT-1 Research Initiative would be that alterations in the mitochondrial membrane potential can cause Nrf2 activation.

**HIF-1** is a transcription factor that binds to the hypoxia responsive element (HRE), turning on the transcription of a number of genes involved in angiogenesis, glycolysis, and erythropoiesis. Under normoxic conditions, hydroxylation of two proline residues by prolyl hydroxylases promotes interaction with the von Hippel-Lindau protein (pVHL) - ubiquitin E3 ligase complex, leading to HIF-1 degradation. Prolyl hydroxylases require oxygen as a substrate and are therefore inhibited under low oxygen environments. Thus, hypoxia is a major stimulus for HIF1 alpha stabilization. Similar to Nrf2, levels of HIF-1$\alpha$ are increased by alkylating thiol reagents such as NAPQI and acrolein, but via a mechanism that is not yet understood (Olmos et al., 2007; Sparkenbaugh et al., 2011). Unlike Nrf2, however, redox cycling agents (DMNQ or doxorubicin) do not affect the levels of HIF-1$\alpha$, and inhibitors of oxidative phosphorylation (rotenone or oligomycin) reduce the levels of HIF-1$\alpha$ because they decrease oxygen utilization (Dayan et al., 2009; Chua et al., 2010; Pointon et al., 2010).

Thus, characteristic responses to toxicants by the Nrf2 and HIF-1$\alpha$ systems represent a tool to potentially resolve MOAs of toxicity at the molecular level, and monitoring levels of these nuclear factors or their immediate down-stream effectors is recommended for all toxicants studied.

**Nuclear Hormone Receptors (NHRs)**

Screening assays for nuclear hormone receptor activation also demonstrate the high promiscuity of several of these systems (Table 4.6). Based on the profiles below, we propose...
to target the CAR, PXR, LXR, and AhR nuclear hormone receptors for characterization based on a common theme of regulation of lipid and steroid metabolism in hepatocytes. In addition to responding to xenobiotics, these receptors have major roles in cholesterol, bile acid, and fatty acid homeostasis (Rezen et al., 2011). The selection of standards for NHR ligands is still under discussion, but compounds under consideration are listed in Table 4.7.

### Table 4.7 Hit rates from TOXCAST transcription factor screens*.

<table>
<thead>
<tr>
<th>Compound</th>
<th>NHR</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ß-Naphthoflavone</td>
<td>AHR</td>
<td>DRE-dependent induction of metabolizing enzymes and CAR with repression of cholesterol biosynthesis.</td>
</tr>
<tr>
<td>3&quot;,4&quot;-Dimethoxy-a-</td>
<td>AHR</td>
<td>DRE-independent repression of cytokine mediated acute phase response.</td>
</tr>
<tr>
<td>naphthoflavone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GNF361</td>
<td>AHR</td>
<td>Antagonist of both DRE-dependent and –independent activity.</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>CAR</td>
<td>Induction of metabolizing enzymes and epigenetic alterations of DNA. Mechanism of CAR activation not clear.</td>
</tr>
<tr>
<td>T0901317</td>
<td>LXR</td>
<td>LXRα/ß nonselective agonist induces lipogenesis, steatosis, and secretion of LDL.</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>ERa</td>
<td>Causes hepatic steatosis <em>in vivo</em>. Primary interest is for epigenetic modification of DNA.</td>
</tr>
</tbody>
</table>

* The compound selection strategy for NHRs is still under discussion.

**CAR/PXR.** Many adverse events associated with NHRs are carcinogenic and/or teratogenic and would be outside the scope of the SEURAT-1 Research Initiative. However, a view is emerging that the MoA for these adverse events derives in many cases from dysregulation of lipid and steroid metabolism in the liver or other tissues of interest to the SEURAT-1 Research Initiative, which makes these NHRs relevant to compound selection. This view is exemplified by the triazoles, which produce an increase in serum testosterone which in turn results in developmental, reproductive, and carcinogenic adverse events. An analysis of gene expression profiles shows that the increased testosterone is due to dysregulation of lipid and cholesterol metabolism in the liver, mediated via the CAR and PXR receptors (Goetz & Dix, 2009a).

The gene expression profiles for the triazoles are more coherent in mouse than in human hepatocytes (Goetz & Dix, 2009b), making them less useful for the SEURAT-1 Research Initiative. Phenobarbital is a prototypical CAR activator that does have well-characterized activity in human hepatocytes and is of special interest because it causes epigenetic changes on repeated dosing in mice (Lempainen et al., 2011; Gerets et al., 2012). Phenobarbital is not a CAR ligand, however, and the mechanism by which it activates CAR is not clear (Zelko et al., 2001).
PXR is promiscuous but species-specific in its ligand binding, and specific monitoring of PXR activation for all compound standards is desirable (Mottino & Catania, 2008). Gene expression profiles from activation of PXR by rifampicin have been described, making rifampicin a desirable reference compound (Gerets et al., 2012).

**AHR.** Persistent activation of AHR receptor is the source of toxicity for the archetypical dioxin class of teratogenic and carcinogenic toxicants (Birnbaum & Tuomisto, 2000). Significant non-carcinogenic adverse events are associated with this receptor, however, and the liver is a major (though not the sole) target organ, with liver hyperplasia, fatty infiltration, and necrosis reported in multiple species (Birnbaum & Tuomisto, 2000). The classical mode of activation involves binding of ligand to AHR, which then migrates to the nucleus and associates with the dioxin response element (DRE) (Petrulius & Perdew, 2002). Known responses to AHR activation are increased levels of selected phase I and II metabolizing enzymes and modulation of levels or activities of CAR, PXR, ERα, NFκB, and TGF-β1 (Patel et al., 2007). Down-regulation of cholesterol biosynthesis is a constitutive activity that is enhanced by ligands (Tanos et al., 2012). In addition, in depth analysis of the SAR for AHR ligands has revealed a second activation mode that mediates the acute phase inflammatory response but does not involve DRE (Murray et al., 2011). This SAR has also identified antagonists for DRE-dependent and –independent pathways (Smith et al., 2011).

**LXR** receptors are relatively promiscuous and are also of interest in the regulation of cholesterol and lipid metabolism. LXRα and LXRβ are distinguished by differences tissue expression patterns but so far have not been distinguishable with respect to ligand selectivity (Osterveer et al., 2010). LXRs were proposed as drug targets for prevention of coronary atherosclerosis, since agonists have been proposed to increase the expression of the ABCA1 cholesterol transporter, increasing the circulation of HDL. However, it was found that agonists also cause an increase in fatty acid biosynthesis with concomittant steatosis, making their utility questionable (Schultz et al., 2000; Collins et al., 2002; Grefhorst et al., 2002).

**ERα.** Tamoxifen is currently being evaluated within the SEURAT-1 Research Initiative experimentally as a reference standard hepatotoxin based on its well-characterized gene expression and epigenetic profiles, and we are awaiting preliminary experimental results before deciding on this compound. However, while tamoxifen causes a high incidence of steatosis in the clinic, this may not be a direct effect, since steatosis is not observed for hepatocytes in culture at concentrations relevant to in vivo exposures (Donatoa et al., 2009; Moya et al., 2010). This observation raises a cautionary flag that responses to steroid metabolism in
particular may be mediated by extrahepatic systems that will not be reflected in cultured cells. This question will be investigated more extensively before final recommendations for nuclear hormone receptors are made.

MoA Standards for Oxidative Phosphorylation

**Rotenone:** Rotenone is an inhibitor of complex I of the electron transport chain and thus represents one of the possible MoAs of cytotoxicity for the archetypical liver toxicant, acetaminophen. Inhibition of complex I is also a common MoA for pesticides (Sherer et al., 2007). More generally 'mitochondrial dysfunction' is a focus for understanding unpredictable drug-induced injury in multiple tissues, and multiple MoAs that lead to mitochondrial dysfunction are linked to inhibition of oxidative phosphorylation (Labbe et al., 2008).

Inhibition at any point in the oxidative phosphorylation pathway blocks oxidation of NADH and switches cells from aerobic to anaerobic metabolism; however, most cells in cell culture have enough capacity for anaerobic glycolysis to survive (Brand & Nicholls, 2011). Since oxidation of NADH is blocked, the reduction potential of the cell is not depleted. Inhibition by rotenone, however, also blocks formation of the mitochondrial proton gradient. This gradient is used to drive processes in addition to oxidative phosphorylation, for example homeostasis of mitochondrial Ca<sup>2+</sup>. Increased free radical formation is also associated with inhibition of the electron transport chain (Brand & Nicholls, 2011). Rotenone is included as a compound standard so that inhibition of complex I can be addressed explicitly as an MoA of toxicity.

**Oligomycin:** Oligomycin is a specific inhibitor of ATP synthase in the oxidative phosphorylation pathway. Like rotenone, the reduction potential of the cell is maintained, but unlike rotenone, the mitochondrial proton gradient is also maintained. This enzyme complex is, perhaps surprisingly, promiscuous to inhibition, with 4 of 20 mitochondrial disruptors examined in a recent screening assay showing inhibition (Nadanaciva et al., 2007). One of these inhibitors was tamoxifen, which also inhibited complex II + III and complex IV of the electron transport chain. Given this broad spectrum of activity for a hydrophobic drug such as tamoxifen and the discussion above concerning the modes of action for chlorpromazine and amiodarone vs. oxidative phosphorylation, we suggest that ATP synthase is a sensitive indicator of membrane disruption. Thus, this key metabolic enzyme is included in the category of promiscuous receptors based on the observation of antagonism at a high frequency, with a possible contribution of membrane disruption to this promiscuity. This MoA for disruption of mitochondrial function is intrinsically different from alkylating and redox cycling MoAs.

The effect of oligomycin is essentially to shift cells to anaerobic glycolysis. Although oligomycin is a potent acute toxin in vivo, cells in culture generally have sufficient capacity for glycolysis to survive (Brand & Nicholls, 2011). Thus, oligomycin will act as a repeated dose toxin under
high glucose cell culture conditions and should cause adaptive cellular responses without acute cytotoxicity.

**FCCP (Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone):** FCCP is an uncoupler of the mitochondrial membrane potential (*Brand & Nicholls, 2011*). It is more specific in its actions and therefore preferred as a reference standard over the more common dinitrophenol. The structural requirement for this class of mitochondrial disruptors is essentially a cationic or anionic amphiphile with \( pK_a \) near neutrality, making it an archetypical promiscuous ligand type. Relative to other inhibitors of oxidative phosphorylation, this MoA is unique in depleting NADH. The effect more closely resembles redox cycling by compounds such as DMNQ.

**MoA Standards for Lipid Metabolism**

Lipid accumulation – steatosis, cholestasis, and phospholipidosis – is a common component of toxicity. These effects are commonly associated with chemically reactive and promiscuous toxicants, which obscures the evaluation of phospholipidosis and cholestasis, for example, as protective, as an additional toxicity, or as a benign reaction to a xenobiotic (*Chatman et al., 2009; FDA, 2011; Padda et al., 2011*). Thus, in this section we have selected compounds that, to the extent possible, have minimum complicating additional reactivities, with the purpose of assessing the relevance of long-term exposure to accumulated lipids in human toxicity. To the extent that lipid accumulation turns out to be a benign adverse event, these standards will be negative controls.

**Bosentan:** The bile salt export pump (BSEP) is responsible for exporting bile salts from hepatocytes to the bile. Inhibition of this transporter is therefore a point of intervention that can cause cholestasis with minimal additional consequences. An *in vitro* screen of 200 drugs for BSEP inhibition found that 16% of the compounds tested had IC50's less than 25 μM (*Morgan et al., 2010*), i.e. this transporter is relatively promiscuous. From these compounds we selected bosentan as a standard that is known to cause cholestasis in humans (*Fattinger et al., 2001*). Bosentan is a dual ETA and ETB endothelin receptor antagonist. Since these receptors are not expressed in hepatocytes, bosentan will have minimal confusing effects via its pharmacological mechanism, and there is no evidence that bosentan is metabolized to a reactive intermediate (*FDA, 2001*). To the limit of our current knowledge, therefore, the effect of this compound on hepatocytes in culture should be a result of BSEP inhibition alone.

**Dirlotapide:** Dirlotapide is a microsomal triglyceride transfer protein (MTP) inhibitor that is designed to block the assembly and release of lipoprotein particles into the blood stream.
Selective inhibition of this process in the gastrointestinal tract is a therapeutic target for obesity, while inhibition of MTP in the liver leads to steatosis. Dirlotapide shows sufficient selectivity for use in treating obese dogs but did not prove to have sufficient selectivity for the intestinal tract for use in humans and is therefore suitable as a reference compound for inhibition of MTP in human hepatocytes (Robinson et al., 2011). Although there is insufficient clinical data to fully assess possible off-target effects of this compound, no reactive metabolites have been reported; and the IC50 reported for inhibition of MTP in HepG2 cells is only 1.5 nM, making it possible to use the compound at low concentrations and thereby minimize possible unexpected activities (Li et al., 2007).

Fluoxetine: Drugs that cause phospholipidosis are generally amphiphilic cations that physically associate with and accumulate with phospholipids in lysosomes (Anderson & Borlak, 2006). Fluoxetine is recommended as a standard for phospholipidosis because it is a serotonin reuptake inhibitor with no pharmacological activity in the liver, without a known reactive metabolite, and known to cause phospholipidosis in humans as well as in cultured cells (Gonzalez-Rothi et al., 1995; Nioi et al., 2007). Fluoxetine may not be appropriate for studying phospholipidosis in cardiac cells, however, because it has off-target hERG antagonism activity (Thomas et al., 2002).

Non-MoA Based Selections

Fibrosis: Methotrexate is a dihydrofolate reductase inhibitor that acts primarily to block DNA synthesis by inhibiting conversion of dUMP to dTMP (Kremer, 2004). It is selected as a representative anti-metabolite. Dihydrofolate reductase is not promiscuous in its ligand binding so that this MoA is not expected to be relevant across a broad chemical space. However, liver fibrosis is an adverse event for methotrexate, and this compound is selected as a compound with a well-defined MoA to aid in establishing in vitro models of fibrosis.

Other compounds that cause fibrosis are described with the reactive compounds: CCl₄ and allyl alcohol.

Cardiac function: Cardiomyocytes have historically been difficult to establish and maintain in cell culture, and it is desirable to closely monitor cell lines for the proper phenotypes. We have, therefore, identified reference compounds that can be used to confirm the proper response to key cardiac signalling pathways: epinephrine as an adrenergic agonist; carbachol as a cholinergic agonist; and nifedipine as an L-type Ca channel blocker.

Electron cryomicroscopy: Electron cryomicroscopy is being pursued as method to assess
changes to cellular structure at high resolution. Hygromycin B is a generically toxic ribosome disruptor that is known to cause detectable changes in ribosomal structure and will be used to validate this methodology.

**Epigenetics:** The strategy for identifying reference standards for epigenetic changes is under discussion. The question is open whether to include compounds such as HDAC inhibitors (vorinostat) or DNA methyltransferase inhibitors (decitabine) which are designed specifically to cause epigenetic changes.

**Outlook**

A table summarising the selection of reference compounds is given elsewhere in this book (see working group report of the Gold Compound Working Group, chapter 4.10.3). The list comprise so far reference compounds addressing hepatic and cardiac toxicities. The list will be further extended and will finally include also reference compounds addressing neuronal, muscle and skin toxicities.

### 4.7.3 The ToxBank Data Warehouse

**Background**

The ToxBank data warehouse (TBDW) provides a web-accessible shared repository of know-how and experimental results to support the SEURAT-1 Research Initiative in developing a replacement for in vivo repeated dose toxicity testing. The information within the TBDW is uploaded from the research activities of the cluster partners as well as relevant data and protocols from other sources, such as public databases. The data is collected to enable a cross-cluster integrated data analysis leading to the prediction of repeated dose toxicity. The warehouse will continue to provide access to this knowledge after the SEURAT-1 Research Initiative completes, for both academic and industrial uses.

**Approach**

Prior to designing the TBDW, the ToxBank consortium implemented a detailed requirements gathering exercise. As part of this process, ToxBank partners visited around 20 partners’ sites and conducted interviews with ca. 50 individual scientists covering all consortia. These discussions covered a variety of activities including cell differentiation, cell engineering, biomarker identification, dose response analysis, toxicity testing, ‘-omics’ experiments, chemical analysis, and cell banking. The interviews focused on understanding and recording in detail what specific steps were performed across a variety of tasks. This type of analysis
can only be accurately recorded by observing the actual work. Detailed notes were taken along with examples of documents used. This information was collected to ensure any system design both meets the needs of scientists across the entire cluster at the same time as fitting within current workflows. The interviews, along with other requirements gathering exercises, resulted in over 1,000 separate notes and 40 tasks outlined.

Once the majority of interviews were completed, the ToxBank consortium met in Milan, Italy over three days. A hierarchical view of all notes was constructed as a group on the wall during the first day and the use cases were reviewed. The second day focused on analyzing this information to generate design ideas based on this user data. Priority for these ideas included suggestions that addressed common issues across the cluster or that resulted in the elimination of steps for different tasks and improving the productivity of the task. Based on this analysis, it was possible as a group to prioritise issues and ideas and develop an outline for the TBDW through exploration and evaluation of multiple scenarios. This outline was translated into a series of storyboards that showed the step-by-step process of how scientists would interact with the proposed system and ultimately to a paper prototype to be used to gleam additional requirements. This process is shown in Figure 4.56. This paper prototype was then used in a series of face-to-face meeting with cluster partners who tried to ‘use’ the paper prototype to accomplish the supported use cases. This process uncovered further issues that needed to be addressed and the system was redesigned based on this input.

**Figure 4.56** Using notes from the interviews with SEURAT-1 partners to generate a system design.
ToxBank use cases

The following section summarizes the TBDW, based on three use cases: (1) uploading of protocols (the term protocol will be used in the design of the TBDW to encompass research protocols and Standard Operating Procedures, SOPs), (2) uploading of data, and (3) searching the information.

(1) Use case: uploading of protocols. Once a new protocol has been developed, documented (as a PDF or Word file), and reviewed within the partner’s organisation, it should be uploaded to the TBDW by the Principal Investigator. ToxBank will provide guidelines concerning the content and organisation of this document; however, the system will not impose any restrictions prior to loading. The protocol will be loaded through the ToxBank GUI where additional information will be entered and associated with the protocol. This includes summaries of the protocol, identification of the protocol’s owner, authors of the protocol, and a specification of who should have access to the protocol. In addition, standardised keywords will be assigned to support searching and linking the warehouse to other resources including the Gold Compound or Biomaterials wiki.

(2) Use case: uploading of study data. The data are loaded in a similar manner; however, a protocol must have already been loaded that defines how the data at each step was generated. The data should be in a defined and standardised format agreed across the cluster.

Once any new protocols or data are loaded into the system, a regularly scheduled email alerting scientists across the SEURAT-1 Research Initiative who have registered an interest in a specific type of information is sent out.

(3) Use case: searching the information. The protocols and study data loaded can be accessed via a simple free text search. This will return summaries of any information matching the query. The protocols or data can then be viewed or downloaded directly along with links to related information, such as the Gold Compound wiki. Comments can also be sent to the Principal Investigators who loaded the protocols or study data. Where the investigator does not have permission to view the specific protocol or experimental data, only the summary information will be displayed. The investigator is then free to contact the Principal Investigator who loaded the content to request access rights. ToxBank will provide documents to support any bilateral agreements between the two parties. Once an agreement is in place, the Principal Investigator who loaded the information would modify permission levels accordingly.

Figure 4.57 provides a schema for the overall proposed vision for the TBDW.
Advantages of this approach include:

- The focus on managing protocols would impact a large number of users across the SEURAT-1 Research Initiative
- Protocol development is close to current work activities, especially the SEURAT-1 focus on experimental development
- It will provides access to other labs’ protocols
- This approach will link public databases and in-house data
- It will capture the best conditions (the experiment may have gone through an optimisation process)
- The use of standardised data templates will enable later integrated analysis
- It provides early access to protected data for specific individuals or groups (under a bilateral agreement)
- It provides access to consistently formatted data for data mining
- It is a flexible approach allowing for data loading at different times
- It provides access to raw data to support valuable results and potentially later re-analysis

The focus of the first phase of the ToxBank project is the development of the unified data
access. As this is being implemented over the next year, the ToxBank consortium will continue to collect requirements and conduct collaborative research related to the direct access to '-omics' data and integrated data analysis, to be implemented as phase 2 of the project.

The ToxBank Data Warehouse System

Once logged in, a user will arrive at the primary ToxBank screen, from which the investigator will be able to access the contents in the warehouse or upload new information or documents; however, it should be noted that only Principal Investigators will be able to upload protocols or data generated within an investigation.

![Main ToxBank Data Warehouse User Interface](image)

*Figure 4.58 Main ToxBank Data Warehouse User Interface.*

From this main screen (*Figure 4.58*), investigators will be able to upload protocols and study data, define email alerts, and search the warehouse using a free text search.

In each laboratory, the Principal Investigator will have responsibility for reviewing and uploading the protocols. Any questions from other investigators of the SEURAT-1 Research Initiative concerning the protocol, as well as requests for access to the protocol (where the protocol has restricted access through ToxBank), will be directed to the Principal Investigator. Guidelines will be made available through the TBDW describing a preferred organisation of the components of a protocol, as well as other topics to be addressed when writing the protocols. Once a file (Word or PDF) has been created that describes a protocol it should be uploaded to the TBDW. From the 'My uploads' page, a link is available 'Upload Protocol' to load a new protocol into the TBDW (*see Figure 4.59*).
Figure 4.59 Data and protocols previously loaded.

This selection results in a new window where the focus is to select and describe the new protocol (see Figure 4.60).

Figure 4.60 Uploading a new protocol.

This process locates the protocol file and additional information is provided that will be presented to anyone who searches the TBDW. The following information must be provided: the protocol file, the protocol title, an abstract, the protocol status (research protocol or standard operating procedure), the consortium who developed the protocol, the authors, the protocol owner, access level, and keywords.

When a new protocol is uploaded into the system it is automatically assigned a new version
number (initially v1). If the protocol is updated at a later date, it is assigned a new version number automatically. All versions of the protocol will be archived and accessible unless a Principal Investigator decides to delete a protocol; however, it will not be possible to delete a protocol where investigation data has been generated and loaded. The Principal Investigator can also create a new version or update information or access levels associated with a protocol. E-mails will be sent to the Principal Investigator annually to check if there are any updates to the protocol or associated metadata.

Data generated as part of an investigation can also be uploaded in a similar manner. Protocols describing the generation of the data at each step of an investigation should have been uploaded and available within the TBDW. These protocols will contain a description of the number and type of data files to be generated. The format should be the ISA-TAB file format (isa-tools.org) generated using a SEURAT-1-configured ISAcreator tool, available for download from the TBDW. Where a template is not defined for a particular type of investigation, the ToxBank scientists will work with the project’s investigators and/or Principal Investigators to define a template that can be used across the entire cluster. The use of these templates is important to ensure that minimal and consistent information is collected across the cluster and essential for combining the data to support an integrated data analysis. The ToxBank GUI will provide a guidance document describing important sections for this file from the ‘Help’ link on the main page.

The protocols and investigation data generated in the SEURAT-1 Research Initiative, as well as related public data, will be accessible through a simple search interface (see Figure 4.61).

![Figure 4.61 Searching the ToxBank repository.](image)

The search engine will identify relevant protocols or investigation results using the associated keywords and summary information, synonym dictionaries and text within the loaded documents. The results are presented as a list, with items ordered according to their relevance to the search query (see Figure 4.62).
This information can be browsed or filtered using any associated keywords as well as consortium information. The protocol or investigation data will be available directly from this result list via the hyperlinks, where all summary information is presented alongside the full protocol or investigation data files, which would be available for downloading (see Figure 4.63). To view protocols and investigation data with a protection level set to SEURAT-1 partners or more restrictive, the investigator will have to be authenticated and authorised to have permissions to access this information. Links to related information such as the ToxBank Gold Compound or Biomaterials wiki will be provided.
Systems Design

The ToxBank system consists of a set of web services, providing access to protocols and data, a search service, and a Web GUI application, offering user-friendly access to the above functionality. The web services, currently developed by partners in Java and Ruby programming languages could run on the same machine, or on geographically dispersed servers, and communicate via the Internet. ToxBank currently adopts the OpenTox framework design, based on the following technological choices (i) the REpresentational State Transfer (REST) software architecture style allowing platform and programming language independence and facilitating the implementation of new data and processing components; (ii) a formally defined common information model, based on the W3C Resource Description Framework (RDF) and communication through well-defined interfaces ensuring interoperability of the web components; (iii) Authentication and authorisation, allowing defining access policies of REST resources, based on OpenAM.

To provide interoperability, ToxBank has selected a number of Open Standards that everyone can freely use, allowing a wide adoption of the ToxBank platform. Standards have been selected to represent data and metadata, both in format as well as in meaning, for machine-to-machine communication, and for interaction with users. Many standards have not been selected just for this particular project, but have been previously adopted by the scientific community. By using Open Standards, ToxBank enables a quick and efficient adoption of its data warehouse technologies.

Data access and upload procedures are defined by the Investigation API. Data is uploaded in ISA-TAB format; data queries are performed with the SPARQL query language. REST operations are available for accessing individual investigations, studies, assays and data files.

Investigations/Studies/Assays/Data are modeled according to ISA-TAB standards as follows:

- **Investigation**: High level concept to link related studies.
- **Study**: The central ISA-TAB unit containing information on the subject under study, its characteristics and any treatments applied.
- **Assay**: Tests performed either on material taken from the subject or on the whole initial subject, which produce qualitative or quantitative measurements.
- **Data**: OpenTox Datasets, tab-separated spreadsheets or external files in native formats (e.g. Affymetrix CEL files).

Investigations are created by submitting a zip archive with files. Individual studies, assays and data files can be submitted as tab separated ISA_TAB files (studies, assays) or as native data files (e.g. Affymetrix CEL files). Alternatively ISA-TAB files can be submitted as Excel files.

This approach to representing the data was selected since the ISA-TAB format will represent
any experiment, including the experimental factors and links to the raw or processed data in their native format. There are also a series of freely available tools to support its use.

Searching within the ToxBank system is provided as a separate web service that is deployable within an existing web container or as a stand-alone application. It was developed using Java and various open source technologies including Restlet and elasticsearch. The search service uses a REpresentational State Transfer (REST) software architecture style fitting well with the overall open design used in the ToxBank system. All services provided by the search subsystem are exposed via a restful interface.

4.7.4 **Innovation**

The selection of candidate reference compounds is key for the success of the SEURAT-1 Research Initiative, as the overall strategy is to follow an Mode of Action approach. To cope with this challenge, we initiated a cross-cluster working group, in which we followed a very stringent selection strategy by linking the available knowledge from existing research on toxicological Mode of Action with chemical reactivity. The toxicological Modes of Action were categorised based on the chemicals reactivity, i.e., compounds that show strong, irreversible interactions with intracellular targets were distinguished from those that show weak, reversible interactions. Most of the in such a manner selected compounds are drugs, as they may possess the same mechanisms as other chemicals in the organism, but most of the human data are available on drugs (from clinical chemistry). Drugs are therefore much more data rich, while it is assumed that the chemistry and biology as related to toxicity would be the same. Even though xenobiotics will very likely not be exclusively related to just one possible toxicological pathway it is reasonable to start with chemicals that are as specific as possible in order to first identify the pitfalls of the general approach, and to be then in the best position for finding solutions.

Regarding the implementation of the ToxBank Data Warehouse, considerable resources were devoted to understanding the needs of the investigators across the entire SEURAT-1 Research Initiative, which focused the system design on common issues. Many alternative approaches were critically evaluated before arriving at the solution outlined here. The solution supports the management, sharing, registration, and version control for all procedures across the SEURAT-1 Research Initiative, along with data generated from investigations. The information is being carefully collected to ensure cross-cluster consistency and documentation that will be essential for an integrated data analysis leading to the prediction of repeated-dose toxicity. The system is also flexible, allowing for access via a simple UI as well as through well-defined APIs. The technical plan makes extensive use of open standards to support future integration with other approaches. The next step is to use the system as a series of pilot projects where improvements will be made to the TBDW before being rolled out to the entire cluster. This TBDW has been carefully designed to support the needs of the SEURAT-1 Research Initiative and future SEURAT clusters.
4.7.5 **Cross-cluster Cooperation**

The selection of reference compounds, as well as the development of the ToxBank Data Warehouse represent an important tool of collaboration and interaction between the different projects of the SEURAT-1 Research Initiative. Thus, these activities have an additional value, beyond the scientific aspect. For the same reason, the positive outcome of these activities represents a success not only for ToxBank, but also for all involved partners of the SEURAT-1 Research Initiative.

4.7.6 **Meetings and Events in the First Year**

The ToxBank consortium has held a series of internal general assembly and interim meetings to discuss across all partners plans and outstanding issues. This included a three day meeting in June 2011 in Milan, Italy where the overall content and structure for the ToxBank data warehouse was mapped out as well as a meeting in August 2011 in Munich, Germany, where details of the technical implementation were outlined. Throughout the year, regular meetings were also held with cluster partners through the data analysis and the Gold Compound working groups as discussed in Sections 4.10.2 and 4.10.3 as well as discussions on the biological materials requirements. This included a series of workshops held at the SEURAT-1 annual meetings on compound selection, integrated data analysis and cell culture. In addition, regular virtual project meetings were held concerning the development of the ToxBank data warehouse and wikis.

4.7.7 **Expected Progress within the Second Year**

The ToxBank data warehouse will be initially rolled out to a small number of SEURAT-1 users as part of a pilot programme or alpha test to ensure the system is easy to use and supports operations across the cluster. Feedback from this initial test will be used to modify the functions of the data warehouse. Once these changes have been implemented, the warehouse will be provided to a larger group of users as part of a beta test. Once this is completed, all SEURAT-1 investigators will be provided access to the warehouse. The ToxBank project will work with SEURAT-1 scientists to load protocols and data, establish guidelines, and develop data templates for any experiments where one does not exist. The keyword hierarchy or glossary will also be extended in consultation with the cluster. A data access agreement will be put in place. The ToxBank team will also integrate relevant experimental data and protocols from outside the cluster and develop case studies illustrating integrated data analysis over public and SEURAT-1 data.

The Gold compound standards will be expanded as necessary including the selection of hepatotoxicity standards with inclusion of nuclear hormone receptor ligands and the selection of
nephrotoxicity standards. The neurotoxicity team will be advised on the design of experiments and selection of reference compounds.

In close collaboration with the stem cell working group, a framework for a new biomaterials wiki will be developed, which will contain information on cells (stem cells, hES/iPS-derived cells, primary cells), reagents (e.g. antibodies, growth factors) and suppliers along with a discussion forum. This wiki will be integrated with the European Human Embryonic Stem Cell Registry or hESCreg database (http://www.hescreg.eu/).

4.7.8 Future Perspectives

The ToxBank project will continue to develop and extend information resources on biological materials and standard chemicals to use throughout the SEURAT-1 Research Initiative in supporting the research activities. The data warehouse will house and provide access to all protocols and experimental data generated through the SEURAT-1 research programme as well as relevant public information. As the project develops, it will be important to support the needs of three distinct communities: those researchers developing the new testing approaches, international regulatory authorities and end users from industry.

From the researchers perspective, it will be critical to develop and integrate workflow, data mining, visualisation and analysis tools to fully support an integrated data analysis. An important component is the reuse and development of relevant core toxicology and neighbouring biological, chemical and medical ontologies. This is essential for the effective handling of data originating from multiple sources, for providing interoperability between different systems, and supporting the integration of diverse toxicology, '-omics', clinical and molecular data into integrative models reflecting biological models, paths and mechanisms. This is important for the use of Linked Resource or Semantic Web approaches used in many current research activities and will ensure those developed are fully interoperable with many other life science projects. Assimilating and interpreting information on the genetic profiles and other data from clinical research will be an important future component of the research.

The development of well-documented and valid approaches will be essential for the regulatory community in accepting any new safety assessment methods. As these research methodologies progress towards industrial prototypes, the needs of the regulatory communities around the world should be consulted to facilitate adoption of these new approaches.

From the industrial perspective, it will be important to understand their need for chemical standards, biological materials, information and analysis tools. This will allow for the development of decision support systems that integrate in vitro and in silico approaches to support risk assessment in research and development. This will be coupled with more formal
purchasing routes for biomaterials and compounds qualified by ToxBank/SEURAT, with broader links to other existing and developing online resources (analysis tools and database systems). This wider service could eventually be established as a sustainable public version of ToxBank not just an internal service to the SEURAT-1 Research Initiative.
References


Recent key references of project members (last 3 years) related to the field of research


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

Bruno Cucinelli

4.8.1 Introduction

COACH is a coordination and support action of the FP7 HEALTH programme that started on 1 January 2011, at the same time as the six research projects of the SEURAT-1 Research Initiative (presented in the previous sections).

The main role of COACH is:

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

COACH provides a central scientific secretariat to the SEURAT-1 Research Initiative (the ‘COACH Office’), organising the cluster level interactions and activities and being the main entry point at the cluster level for all organisations, including the funding organisations, i.e. the European Commission and Cosmetics Europe and all external organisations which are searching contact with this initiative (Figure 4.64).
All the seven projects of the SEURAT-1 Research Initiative are governed by a contractual framework composed of a contract with the European Commission (the FP7 Grant Agreement) and a contract with the cosmetics industry association Cosmetics Europe (previously named Colipa) signed with each of the project consortia. These contracts define 18 month work periods (reporting periods). The first work period is just about to be completed.

The following sections will highlight some important aspects of this first period (summarised in Figure 4.65).

- Establishment of the cluster modus operandi: Scientific Expert Panel (SEP), COACH-office, annual meetings, e-collaboration tools, ...
- Definition of the SEURAT vision and long term research strategy
- Creation of dissemination material and dissemination channels
- Publication of the first SEURAT-1 Annual Report
- Cross-cutting working groups and workshops
- Development of a cluster level training network

Figure 4.65 Main cluster-level achievements of the SEURAT-1 Research Initiative in the first period.
4.8.2 Cluster Level Coordination

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

The start phase of SEURAT-1 can be considered successful. The important achievements of the first work period that can be highlighted include:

- The initiative established the organisational structure, means and tools to support the collaboration between the participants

- The scientific secretariat of the SEURAT-1 Research Initiative (the ‘COACH Office’), has been performing efficiently. The composition of the consortium and share of tasks between the partners has proven to be appropriate. The partners have a complementary background and expertise, which proved to be perfectly in line with the share of responsibilities and tasks of each partner, and in line with the requirements of the research initiative.

- A Scientific Expert Panel (SEP) was established from the start of the cluster (the first constituting SEP meeting was held shortly before the official start of the initiative, in November 2010) and ensured the cluster level strategic leadership of the initiative. In average SEP meetings were held every three months, either as physical meeting or as telephone conferences.

The SEP is composed of the coordinators of the six cluster research projects and external experts. The SEP composition is shown in Table 1.1 in the Introduction of this Annual Report.

The SEURAT vision and long term research strategy has been described and published. The research strategy, adopted by the Scientific Advisory Panel on 1 July 2011, was based on a discussion paper prepared by COACH partners ‘University of Tübingen’ and ‘Joint Research Centre’. The strategy describes how the SEURAT-1 Research Initiative wants to achieve the long term target of replacing animal testing in human safety assessment, the global research target SEURAT-1 and beyond. The SEURAT strategy was published in the first volume of the SEURAT-1 Annual Report, issued in September 2011, and will be continuously updated (see also chapter 3).

The collaboration with related research initiatives and institutions in and outside Europe was considered important since the start of COACH. Links were in particular established with:
AXLR8 (Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development), EPAA (The European Partnership for Alternative Approaches to Animal Testing), Tox21 and ToxCast (an initiative of the U.S. Environmental Protection Agency to develop ways to predict potential toxicity and to develop a cost-effective approach for prioritizing the thousands of chemicals that need toxicity testing).

4.8.3 **Facilitating Exchanges between the Participants**

**SEURAT-1** involves about 70 organisations spread over Europe (and some of them outside of Europe) and efficient tools for remote collaboration are key. At the outset of the initiative, **COACH** has set up e-collaboration tools that are being used intensively. Besides dedicated mailing lists, **COACH** is providing a collaborative web platform operated by partner ‘ARTTIC’ which allows to facilitate sharing of information and remote collaboration. The private workspace for the **SEURAT-1** partners is accessible for registered users who are involved in the cluster projects, the European Commission and some experts of Cosmetics Europe who signed a special Non Disclosure Agreement. As of June 2012, there are over 230 individual user accounts and in average about 1,000 pages are visited every month.

The **SEURAT-1** Annual Meetings are the main event for face-to-face meetings of cluster participants. A suitable concept for organising the Annual Meetings was defined and implemented in two meetings (March 2011 and February 2012); the Annual Meetings are basically composed of (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 cluster cross cutting topics, and (iii) a panel discussion drawing conclusions from the discussions and providing an common view on future work orientations and priorities of the research initiative.

Cross-cluster Working Groups were created and focus workshops organised to elaborate common references that are important for a successful collaboration. The cross-cluster working groups are considered as important pillars for facilitating cluster level collaboration. Two Working Groups had been active since the outset of the initiative, focusing on two issues that are key for optimising the research activities and the exploitation or collaborative research results at the cluster level: (i) Gold Compounds and (ii) Data Analysis. These Working Groups were actively supported and followed up by **COACH** partner ‘University of Tübingen’. To support these cross-cluster work activities, **COACH** partners ‘Joint Reserach Centre’ and ‘University of Tübingen’ also prepared workshops organised during the Annual Meetings as breakout sessions and as focus work meetings at JRC facilities in Ispra, Italy (further details are given in chapters 4.9.4 and 4.10, respectively).

In the SEP meeting of February 2012, the importance of the Working Groups for addressing
cluster level issues was underlines and the need for defining a common approach to the organisation of these Working Groups. One result of the second Annual Meeting was the creation of four additional working groups with focus on: (iii) Stem Cells, (iv) Biokinetics, (v) Mode of Action and (vi) Safety Assessment. COACH partner JRC made a proposal for the definition of Terms of Reference for these Working Groups that was first discussed with the coordinators of the six research projects and then submitted for agreement to the Scientific Expert Panel. Each Working Group has a clearly defined scope and is coordinated by two appointed co-leaders.

A cluster level training network has been set-up and a common training programme defined. Since the work programme of the six individual research projects had been defined independently from each other, each consortium had defined its own approach to address the identified training needs. COACH took the initiative to homogenise the training activities at the cluster level, by initiating the creation of a training task force composed of representatives of each of the projects and by proposing a common approach to cluster level training activities. As a result of this work, a first SEURAT-1 Summer School was organised in June 2012. Section 4.11.1 provides further details on the training activities and in particular the first cluster-level summer school.

4.8.4 Information Dissemination

In order to ensure a good visibility of this research initiative, suitable dissemination material was created and the suitable dissemination channels have been set up. A consistent visual identity for SEURAT-1 has been developed (logo, colours, layout of printed and electronic dissemination material, website look and feel, etc.) in collaboration with a professional design company. A number of information dissemination support materials have been created and distributed since the start of the initiative, including a leaflet, a poster, a who’s who booklet that were distributed at the occasion of each Annual Meeting (also available as on-line version) and a standard PPT presentation.

A public website was set up and went on-line at the URL www.seurat-1.eu. It presents the research initiative, its background and aims, the cluster projects, the involved partner organisations and promotes the research activities and results in the field of human safety assessment, in particular regarding alternative repeated dose systemic toxicity testing. Dedicated pages present related events, links, publications, job announcements, etc. Also, to promote the objectives and approach of SEURAT-1, the COACH partners participated to a number of scientific events (further details are given in chapter 4.11).

The preparation of the first SEURAT-1 Annual Report was coordinated by COACH partner ‘University of Tübingen’, who proposed the content structure and contributions required. This 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 content. COACH partner ‘University of Tübingen’ collected, reviewed and edited the contributions and conceived the book layout concept in collaboration with COACH partner ‘ARTTIC’ and the appointed professional designer. The first Annual Report was successfully completed by end of September 2011 as electronic version (downloadable from the SEURAT-1 public website). It was printed in about 1,400 copies and distributed over mail and on relevant conferences. A dedicated dissemination channel for the Annual Report was created in the form of a mail list, containing over 500 postal addresses of scientists, experts and stakeholders of SEURAT-1 research results.

4.8.5 Next Steps

The next work topics on the COACH priority list include:

The strategic review of the cluster. Due to the specific contractual construction of the initiative (each individual project has a contract with the European Commission and with Cosmetics Europe, but there is no cluster level contract, defining results and milestones at the cluster level), a method needs to be agreed upon to define the performance of the cluster as a whole. COACH partner ‘Joint Research Centre’ developed a proposal for such a method to be applied by the Scientific Expert Panel (SEP) during its physical meeting on 8-9 June 2012 in Oeiras. The aim will be to analyse the strengths and weaknesses of the initiative at the cluster level and to identify any possibly corrective action required to optimise the functioning of the cluster level collaboration and the achievement of cluster level results. At the time of writing this chapter, the SEP meeting was still under preparation. A summary of the outcome of this strategic review will be described in the next volume of the SEURAT-1 Annual Report.

The cluster level Non Disclosure Agreement and data access agreement. Although the six projects have a self-standing research programme, they are addressing complementary research areas, the basic idea of the cluster is that each of these project is a building block of a large research initiative. The close collaboration and sharing of research results at the cluster level is therefore key to optimise the outcome of the global research work. In order to support the optimisation of the research at the cluster level and the consolidation and sharing of scientific information between the participants, one of the six projects, ToxBank is developing a Data Warehouse. As the individual projects start producing experimental data and the development of the ToxBank Data Warehouse is progressing, it becomes important that the conditions for sharing information at the cluster level are defined in a legally binding agreement. It is not a trivial task to get a legally binding document signed by about 70 organisations. COACH partner ‘ARTTIC’ has been closely collaborating with the ToxBank coordinator to get such an agreement signed in 2011, but this first attempt has not been successful. The objective of the COACH and ToxBank is to get such an agreement signed
before the end of 2012, to allow the cluster partners to share confidential data during the next Annual Meeting.

Promotion of the SEURAT-1 research strategy. COACH will use the publication of the second Annual Report as opportunity to further promote the SEURAT-1 Research Initiative and the research strategy it has developed. The two major events considered for 2012 are the organisation of a round table in September 2012 in Brussels and the ESOF meeting in July 2012 in Dublin (esof2012.org/). This event is a major pan-European meeting, held under the auspices of Euroscience, aiming to showcase the latest advances in science and technology, promote a dialogue on the role of science and technology in society and public policy and stimulate and provoke public interest. Press releases will be published at these occasions. The COACH partners are also planning to submit a short version of the SEURAT-1 strategy for publication in an appropriate scientific journal.

Preparation of the next phase towards the achievement of the SEURAT long term goals. The partners and stakeholders of this research initiative consider that SEURAT-1 is only the first step in a long research effort required to develop alternative solutions for human safety assessment in view to replace animal testing approaches. The SEURAT-1 Research Initiative has started only last year, but it will cover only a period of 5 years and the partners and stakeholders need already to think on how to organise the next phase of this long term research work, i.e. what will be the scope of SEURAT-2, which form of public-private-partnership could be envisaged and how could public and private research funding programmes support the research efforts. The aim of COACH will therefore to stimulate the preparation of recommendations and/or proposals for the definition of future research work orientations and accompanying activities such as certification of the developed technologies and tools, as input to public and private research funding programmes.

Priority work topics for the second period will also address the further development of the achievements made in the first period, i.e. the efficient operation of the Working Groups, the preparation of the next training activities, and the collaboration with related research initiatives and organisations.
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4.9 Project and Cluster Activities

Mark Cronin, Barry Hardy, Elmar Heinzle, Jürgen Hescheler, Marc Peschanski, Catherine Verfaillie

4.9.1 Project and Cluster Activities

SCR&Tox consortium members meet face to face on a 6-month basis and web-conference every 3 months, according to the initial working plan.

Three face to face meetings have taken place: the SCR&Tox kick-off meeting, held in Evry, France on 13 – 14 January 2011. During the kick-off meeting, consortium members have made a presentation on their contribution to the work plan. The second meeting was the SCR&Tox 6th month meeting, held in Gothenburg, Sweden, on 23 – 24 August 2011. The programme was divided into two parts: in the first part, four different round tables were organised on: (i) State of the art of iPSC; (ii) toxicity pathway testing; iii) how can SCR&Tox benefit from other projects? and (iv) technology transfer within SCR&Tox. The second part was focused on advances, with presentations from selected groups on the achievements and challenges, followed by a discussion on future plans. The second SCR&Tox annual meeting was held in Leipzig, Germany on 28 February – 1 March 2012. One representative of each institution highlighted their first year achievements and challenges. Representatives of all work packages were present, including those that will be formally active in the second part of the working program, as well as the members of the external advisory board, who gave a feedback regarding the SCR&Tox first year of performance. Tilman Gocht from COACH attended the meeting and gave a presentation on the SEURAT-1 cluster activities and actively participated in the discussion on how to continue interacting efficiently with other projects of the SEURAT-1 Research Initiative.

Also, web conference meetings are organised every three months, where all the SCR&Tox members from the active work-packages expose their findings and advances, as well as the delays and modifications to the initially established working plan. These web conferences constitute a fruitful resource for the scientific discussion.

In conjunction with its 1st Annual meeting in February 2012, SCR&Tox initiated a first workshop on pluripotent stem cells in toxicology for European and national regulatory authorities as part of its training programme, and is planning to extend this initiated dialogue. The next SCR&Tox mid-term meeting will be held in Ispra, Italy from 6 – 7 September 2012.

HeMiBio: The HeMiBio consortium held its kick-off meeting in January 2011 in Leuven. All
the partners discussed the expertise in their groups and the progress made towards the goals of the proposal. The external advisory board members were also present at the meeting. A second consortium-wide meeting was held in Leuven in June 2011, in this instance, without the external advisory board members. During the meeting, the two subgroups (biology-oriented partners and technology-oriented partners) held breakout sessions to discuss streamlining the studies to be done in the coming months. In addition, we have arranged several monthly web conferences between all partners, as well as focused discussions between the ‘biology’ partners or ‘engineering’ partners.

The second annual meeting was held in Jerusalem in January 2012. Besides discussion about the progress made in the first year, the biology- and technology-oriented partners held the breakout sessions again. The external advisory board members provided very significant feedback related to the progress made and the plans for the second year of HeMiBio. This meeting was preceded by a Winter School, titled ‘Introduction to Microfabrication Technology for Biology and Medicine’. Information regarding the programme can be found at http://www.hemibio.eu/NEWS-EVENTS/training-a-education.html and in chapter 4.11.1 of this book.

Finally, we have developed an internal HeMiBio exchange programme, where students/scientists of one partner can spend a period of time in the lab of other partners to train the students/scientists in a complimentary technology and increase the within-consortium collaboration.

**DETECTIVE**: The DETECTIVE kick-off meeting took place at the University of Cologne on 25 – 26 January 2011. During this first internal meeting of the consortium and the DETECTIVE Advisory Board, initial discussions started about the available cellular model systems, the selection of reference compounds, functional readouts, ‘-omics’ readouts and statistical analysis of biomarkers. A public event in the evening of the first day accompanied the internal project meeting.

Over the course of the first project year, numerous teleconferences and virtual meetings have been organized within the consortium. Four teleconferences were centrally organized by the Knowledge Management with all partners involved in the experimental part of the project, in order to continuously follow up on the work progress (i.e. identification of suitable cell systems for each target organ, quality control, selection of compounds, definition of exposure protocols etc.), to address specific issues encountered and to decide on the following steps. Further in-depth discussions, teleconferences and meetings took place between the partners and, in particular, between the members of the three target organ groups (heart, liver and kidney).

For a successful start of the DETECTIVE cooperation within the SEURAT-1 Research Initiative, several DETECTIVE partners attended the cluster kick-off meeting organized by COACH in Cascais (Portugal) on 1 – 3 March 2011, some of whom contributed with keynote lectures.
The various workshops and teleconferences organized by ToxBank on data management and compound selection were also regularly attended by DETECTIVE partners. DETECTIVE was also present at the second annual meeting of the SEURAT-1 Research Initiative taking place from 7 – 8 February 2012 in Lisbon and contributed with the presentation of five posters related to the work performed in the DETECTIVE project (see below, chapter 4.9.2).

The second General Assembly meeting of the DETECTIVE consortium took place from 6 – 7 February 2012 in Lisbon and focused on priorities for the second project year and the organisation of four specific working sessions.

**COSMOS**: The COSMOS Kick-Off meeting was hosted by the European Commission’s Joint Research Centre (JRC) in Ispra, Italy on 17 –18 January 2011. This enabled all partners to get a better overview of the different parts of, and interactions within, the COSMOS project, and discuss the scientific approaches, first steps to take and planning for the different work packages. Moreover, the KNIME technology, which is an essential part of COSMOS for implementation and dissemination of the models and workflows developed, was introduced to the partners with a hands-on demonstration. Further KNIME user and developer training was provided in two training workshops on 4 – 7 April 2011 in Zurich, Switzerland.

Delegates from the COSMOS partners and the Scientific Advisory Board met on 28 February 2011 in Cascais, Portugal, for the first COSMOS Annual General Meeting to discuss ongoing and upcoming work in plenary and separate work package break-out sessions. In addition to an outline of the project, COSMOS contributed with an overview of challenges and pitfalls in predicting toxicity to the subsequent SEURAT-1 cluster Kick-Off meeting on 1 – 3 March 2011.

On 23 September 2011, a General Assembly meeting was hosted by the Bulgarian Academy of Sciences (CBME-BAS) in Sofia, Bulgaria to update the COSMOS consortium on progress and discuss further planning. This meeting was preceded by a Workshop on Adverse Outcome Pathways (AOP) and Related Technologies on 22 September 2011, introducing and discussing emerging concepts such as AOP, Effectopedia and the use of category formation tools. The aim was to raise awareness of these concepts and associated tools and to develop a strategy/vision for COSMOS, relating to AOP for repeated dose toxicity.

At the end of the first project year, the Second Annual General COSMOS Meeting was held on 6 – 7 February 2012 before the SEURAT-1 Annual Meeting in Lisbon, Portugal. The meeting reviewed the first year results, planned the next steps and discussed specific topics within the work package groups. COSMOS took the lead in the Mode of Action and Biokinetics breakout sessions during the SEURAT-1 Annual Meeting and the respective newly established cross-cluster MOA Working Groups (see below, chapter 4.9.2 and 4.10.4).

COSMOS also contributed to the SEURAT-1 Summer School in June 2012 in Oeiras, Portugal
with an introduction on computational toxicology and mechanisms of action and modules on
data governance, chemical space, the Threshold of Toxicological Concern (TTC) concept and
KNIME workflows.

The next COSMOS General Assembly meeting will be hosted by the University of Bradford on
13 – 14 September 2012, the next Annual General Meeting will be held in conjunction with the
third SEURAT-1 Annual Meeting.

NOTOX: Two NOTOX meetings were held during the year 2011. The first kick-off meeting
was held in January 2011 in Saabücken and was organized by the coordinators (EURICE).
During this meeting, extensive discussions between partners were focused on the cell systems
and the test compounds. The second NOTOX progress meeting took place in Paris in June
2011 with INRIA as hosts. The highlights of these meetings were the following:

► It was decided to start with HepaRG cell line, as it is the best in vitro model
available in terms of drug metabolism capacity and, therefore, its use in
metabolism based toxicity assessment is essential.

► It was also decided to explore and use various organotypic cultivation
techniques which included 3D bioreactors, sandwich cultures and organoid
structures.

► Four test compounds (acetaminophen, valproic acid, tamoxifen and
troglitazone) were chosen at the start of the project. The TOXBANK efforts in
compound selection were highlighted and appreciated.

► Harmonization of protocols was highly stressed. In this regard, the sources
of the medium and other supplements were decided and batches reserved for
NOTOX project. A batch of HepaRG cells was also reserved for NOTOX.

► The involvement of modelers in experimental design was emphasized.

► It was decided to use the NOTOX WiKiplatform for storing experimental
protocols, SOPs and data.

► As a part of NOTOX dissemination strategy, the NOTOX film was
discussed.

The next project meeting is planned in Stockholm from 11 – 12 June 2012, hosted by
Karolinska Institute. The progress of the experiments will be assessed. The data will be used
in establishing and optimizing the multi-scale models not only for biochemical and regulatory
networks but also for spatial-temporal and structural models based on 3D tomography
reconstructions.
4.9.2 **Cluster Meeting of the SEURAT-1 Research Initiative**

*The COACH Team*

The second Annual Meeting of the SEURAT-1 Research Initiative was held on 8 – 9 February in Lisbon, Portugal. Ian Cotgreave, co-chair of the SEURAT-1 Scientific Expert Panel (SEP), opened the meeting and welcomed the participants. He reviewed the messages he had given at the first annual meeting, when he had identified 3 fundamental ‘Cs’ for the progress of SEURAT-1, which were creativity, collaboration and courageousness. For further improvement and success of the research initiative, he further identified additional four ‘Cs’ that he asked the participants to carefully consider: celebrate (what had been achieved so far), consolidate, consider and communicate. He pointed out that the cluster of projects was expected to give an added value compared to the outcome of the individual projects put together, and to achieve this the cluster ‘vehicle’ had to be guided by wisdom and courage.

The Director General of Cosmetics Europe, Bertil Heerink, welcomed the participants of the SEURAT-1 Research Initiative and underlined its the importance for his organisation by describing the difficulties and impossibility of meeting the requirements stated by the Cosmetics Directive to ban all animal tested cosmetic products by 2013. Rob Taalman continued by describing the efforts of Cosmetics Europe to support the development of a non-animal toolbox capable of replacing the need for animal test data covering systemic toxicity. He described the currently ongoing research projects on alternative methods supported by the Cosmetics Europe and informed about the extension of its research programme to find alternatives to animal testing through additional funding of approximately €8 million. Maurice Whelan (COACH – Joint Research Centre) reminded the participants in his presentation of the SEURAT-1 vision and strategy, developed and agreed as a follow-up to the first annual meeting. He defined the four cluster-level objectives, suggested the tactics how to achieve these objectives and how to demonstrate the SEURAT-1 proof-of-concept. He stressed that the only way to gain success is to work together.

The meeting was continued with progress reports from the seven coordinators of the SEURAT-1 projects. Marc Perschanski, SCR&Tox coordinator, highlighted the importance that all partners sign a confidentiality agreement on the cluster level before the next annual meeting, to ensure the possibility of reporting scientific progress and exchanging the data when there would be more substantial results to present, after the second year of activities. So far, the main activities of SCR&Tox were related to the project areas focusing on biological resources and technological resources, respectively. Initial repeated dose toxicity tests were already performed with two types of cells used: human embryo cell lines and human fibroblasts. He stressed that there is a lack of toxicological expertise within the SCR&Tox consortium, and that they welcomed input and advice in discussion with other SEURAT-1 partners to compensate for this.

At this occasion, HeMiBio was presented by Leo van Grunsven. He discussed the progress
made in isolation and culturing of different liver cells as well as the development of sensors as the components to be included in a bioreactor mimicking the functions of a human liver. Furthermore, he informed that HeMiBio had organised a winter school at the Hebrew University in Jerusalem in January, including a substantial part of hands-on exercises with bioreactors, which had been considered very useful to all participants. 

The coordinator Jürgen Hescheler reported back from the first year of activities within DETECTIVE. He asked the audience how many biomarkers would be of interest to test and how many would actually be needed. So far, functional readouts had been made while ‘omics’ results would become available only in the second year. Human pluripotent embryonic stem cells were tested, including cardiocytes, hepatocyte-like cells and neurones. The project partners were divided into three groups: the heart, liver and kidney group. In year two, besides producing ‘omics’ read-outs, the selected gold compounds will be used for further tests, and selection criteria for biomarkers will be set up. Jürgen Hescheler hopes that DETECTIVE would soon have major interactions with SCR&Tox to exchange cell lines and protocols. 

Mark Cronin, COSMOS coordinator, reported the highlights of the project from the 1st year. Tools for data entry and data searching had been developed. The project had agreed on consistent data representation and data sharing. The COSMOS Threshold of Toxicological Concern (TTC) database and the COSMOS Cosmetics inventory had been created in an attempt to define the chemical space of cosmetics ingredients. QSARs for support to PBPK modelling had been investigated. A cell based in vitro model had been developed to predict what in reality is measured in a cell assay, and acetaminophen had been used as a case study to set up a PBPK model to extrapolate rat data to human. KNIME, a user-friendly and comprehensive open-source data integration, processing, analysis, and exploration platform, was used to set up and develop further pathway workflows. 

The NOTOX coordinator Elmer Heinzle focused on the first results of toxicity testing obtained from the bioreactor tests. 3D bioreactors (the jellyfish) had been set up for testing and also the more simplified 3D structure of hepatocyte droplets had been successfully tested. Different cell lines relevant to hepatotoxicity had been exposed to different test substances, to map similarities between the cell lines and compare those to human hepatocytes. Besides interactions with other SEURAT-1 projects, NOTOX has also exchange activities with the German Virtual Liver project. 

The progress in ToxBank was presented by Emilio Benfenati. He explained how data can be uploaded through the ToxBank web page to populate the ToxBank Data Warehouse. A next step would then be for the other projects to test the protocol service and provide feedback to ToxBank. The results from the Gold Compound working group were presented by Jeff Wiseman. He explained to the plenary how the choice of the hepatoxic substances had been made depending on their Mode of Action and asked for advice on how to cover additional relevant Modes of Actions for liver toxicity. It was questioned whether it would be useful to
identify more negative controls. Jeff Wiseman replied that there was a general problem with negative controls, as one usually can not be sure that they are negative at higher doses and it is difficult to decide the realistic dose.

Bruno Cucinelli, the COACH coordinator, reported back on the activities of the coordinating SEURAT-1 project in their first year of activities. He concluded that COACH, with the support of the other projects and the Scientific Expert Panel, had successfully managed to create visibility and recognition for SEURAT-1 as the major research initiative in the field of repeated dose toxicity testing.

In the afternoon of the first day, scientific focus sessions were conducted. The meeting participants were divided into the following four breakout groups, based on their areas of interest:

- Stem cells – characterisation and standardisation (Chair: Glyn Stacey – SCR&Tox)
- Biokinetics – in vitro to in vivo extrapolation (Chair: Jose Zaldivar – COSMOS)
- Mode of Action – repeated dose system toxicity (Chair: Mark Cronin – COSMOS)
- Safety assessment – using alternative methods (Chair: Andrew White – Unilever)

All breakout groups worked around some thought starter presentations followed by a brainstorming session. Subsequently, the final outcome was reported back to the plenary and is summarised in the following.

The discussions in the stem cell breakout session (reported back by Glyn Stacey) had been focused on certain cell models that would be made available via SCR&Tox and how to standardise quality control issues of the cells between the different partners and projects. ToxBank would coordinate not only the compound selection, but also the SOPs to be used within the cluster. A number of cross-consortia cell model subgroups including iPS cell lines, hES cell lines, as well as differentiated progenitors had been identified, and SCR&Tox will keep an updated overview about their use in the different SEURAT-1 projects.

Alexandre Péry (COSMOS) summarised the results from the biokinetics breakout group. He stressed that for any biokinetic modelling activity concentration measurements are key. It is possible to make biokinetic models to assist the other SEURAT-1 projects to design in vitro and bioreactor models. It would be necessary for the modellers to obtain concentration measurements and effects data from the in vitro experiments to be able to extrapolate the result. It was clearly stated in this breakout group that a paradigm shift was necessary from pure experimental approaches to a guided model based approach.

George Daston (Procter & Gamble) reported the outcome of the Mode of Action breakout
The application of the Adverse Outcome Pathway (AOP) framework approach within the SEURAT-1 Research Initiative was supported. It is suggested that AOPs are used as a practical tool to organise Mode of Action information and capture inter-relations in the cell by means of ‘-omics’ and other in vitro data. A special focus will be made to link Molecular Initiating Events to possible adverse outcomes. Dr Daston stressed that it would be necessary to include dose dependencies and to achieve quantitative descriptions of the AOPs.

Andrew White (Unilever) pointed out, when reporting back from the Safety Assessment breakout group, the importance of understanding the regulatory context when setting up a testing system to be applied. For instance, it was assumed that the same type of testing would not be required, for example, for cosmetics as for the pharmaceuticals. It is also important to understand the protection goal. The use of benchmarking based on known mechanistic pathways, as opposed to animal tested, was suggested. He summarised the tasks that had been agreed upon to tackle in the Safety Assessment working group: (i) define what is good enough in a phenotype of cells (not using more complex models than necessary); (ii) examine what would be useful in building confidence in test systems and integrated testing strategies (for example, biokinetic modelling in combination with in vitro results); (iii) work with ToxBank to identify negatives that realistically help define adaptive versus adverse effects.

The issues discussed in all four breakout groups had been of a general cross-cluster interest, and meeting participants volunteered to continue these discussions and carry them forward during the year until the next annual meeting. It was agreed that all four breakout sessions would continue their work as SEURAT-1 working groups. More detailed informations about the breakout groups, as well as their objectives of the newly generated working groups are reported elsewhere (see chapters 4.10.4 – 4.10.7).

The first meeting day was completed by a poster session in the evening, presenting results from the separate projects. The aim of the poster session was to get the possibility to report more details on the different projects than had been possible in the coordinators’ reports in the morning, and also to encourage the participation of young scientists in the cluster at the annual meeting. 30 posters were presented and three out of those were awarded as best posters at the end of the meeting (see below).

The second meeting day was opened by an inspiring presentation given by Mel Andersen from the Hamner Institute (USA). He had been invited as an external key note speaker, to strengthen a good communication and openness between SEURAT-1 and US colleagues working on similar issues, and, of course, due to his exceptional experience in the field of a new safety assessment of chemicals and as a co-author of the pioneering US NRC report ‘Toxicity testing in 21st century – a vision and strategy’. Mel Andersen said that it was time to start understanding biology. He explained that a chemical coming into the cell would disturb one or more normal signalling pathways and if this would lead to an adverse outcome at
the organ or organism level, this would be called an Adverse Outcome Pathway. However, he was not very favourable of this terminology, as it is quite difficult to predict an adverse outcome and it would be necessary to take into account repair mechanisms. He felt more comfortable talking about Mode of Actions. It would be necessary to understand them, but once understood, it would be possible to identify phenotypic anchors that would act as a basis for safety assessment. To start with, case studies using well-studied prototype compounds with known Modes of Actions, are promising. In addition, it is necessary to set up computational systems biology models and to make IVIVE modelling (in vitro to in vivo extrapolation). In his concluding remarks, Mel Andersen stressed the importance of reaching a global application of a new safety assessment paradigm of chemicals, and that it is mandatory to find convincing models to be accepted by a multicultural society with different problems, acceptance and technical infrastructure.

A panel discussion followed Mel Andersen’s key note speech. Michael Schwarz (COACH) proposed the following question for discussion: How can we define adversity at the cellular level? He further described that it would be necessary to identify the possible receptors and then the pathways to be able to read out what is happening in vitro. There are thousands of primary targets possible; however, as we look at the pathways, there will very likely be a limited number of key events, which might be more distinguishable. He suggested that a way forward would be to search for relevant transcription factors by identifying clusters of co-regulated genes (using ‘omics’ techniques). He further said that a lot of in vivo data are available in existing databases that could be mined for the identification of regulatory networks. He made the example of the Japanese OPEN TG-GATEs database (http://toxico.nibio.go.jp/open-tggates/search.html).

George Daston (Procter & Gamble) was the second panel speaker. He said that the paradigm shift often mentioned was that we were about to start a third era in biology. The first era had been catalogisation (up to Darwin), the second era could be characterised by reductionism, however now we are about to enter the third era. We would need to understand multiple insults and multiple targets. It will not be only one target or effect to measure. George Daston stated that we are about to start the era of computational biology. He concluded that predictive toxicology is emerging due to tremendous advances in biotechnology and computational science; the tools enabling these advances were widely available, and a tiered approach would allow for toxicity assessment to become hypothesis-driven, but all these advances would be meaningless without a great deal of expertise in toxicology, chemistry, informatics and molecular biology.

Paul Carmichael (Unilever) stated that the starting point for a new safety assessment must be the exposure - a consumer use assessment. Exposure should be kept in focus, and by starting from the exposure assessment, the most suitable in vitro assays should be chosen. The computational methods of the circuitry of the relevant toxicity pathways would then be key and, together with IVIVE, support risk assessment based on exposures below the levels
of significant pathway perturbations. He concluded that the coordination and integration are the basis for achieving the results as mentioned in the SEURAT vision.

Bas Blaaubroer (Utrecht University) initiated his panel speech by posing the following questions: Are all activities directed towards the common goal? Is the complete programme sufficient to tackle the problem? He encouraged the consortium to try to measure as detailed as possible the concentrations in the different phases of the in vitro systems. He preferred to talk about QIVIVE rather than IVIVE stressing the importance of making quantitative extrapolations. He pleaded to everyone to integrate biokinetic considerations in all parts of the programme. He finalised the speech by stating that we were not really talking about a new era of biology but a new era of science, as we are now merging many fields together in a holistic combined picture. He called this a second renaissance.

Derek Knight (ECHA) talked about regulatory requirements. REACH was made to be good enough – fit for purpose. He pointed out that the door is already open for non-standard data under REACH. There is no legal barrier to use such data. His vision was to use the new test systems in a weight of evidence approach and to consider pragmatically a higher value of the assessment factor in case of higher uncertainty in the prediction. Derek Knight also suggested that the information from the new approaches could be used to strengthen the case for read-across and chemical categories in a weight of evidence approach.

Ian Cotgreave (AstraZeneca) was the last panel member to speak up and he wanted to provoke the audience with a few reflections. First, he asked how we could estimate risk to average people, as there is no average person. Assessment of human risk needs to take into consideration the individuality in response. In some way, human variation must be built into any model used for safety assessment. He continued by questioning how we would be able to predict what would happen when a certain chemical would temporarily disturb different pathways. It would be preferable to approach the problem with some reductionist thinking, as the problem, in most cases, would become too complex. Another remark he made was that in vitro systems cannot reflect real-case in vivo situations, for example, the case of obesity is generally not considered as excessive fat and would not be introduced into in vitro systems. His last reflection was whether it would be possible to identify an ‘average-cell’ representing all cells, rather than trying to understand what is happening in cells originating from different tissues, because most pathways would be present in all cells regardless of the tissue type.

An open plenary discussion followed facilitated by Maurice Whelan (COACH). The discussion focused around the following key aspects:

- Animal testing: It was agreed that first, all in vivo databases should be used, but then some animal testing could become required, in order to complete the picture.
- Adverse Outcome Pathway approach: The integration into risk assessment
was thoroughly discussed and it was clarified that the AOP approach within SEURAT-1 is not contrasting the Mode of Action approach. Rather, the Mode of Action is indeed the core of an AOP, but the AOP also includes other elements such as exposure scenarios and effects on the level of organisms.

⇒ Acceptance and implementation of non-standard methods in the regulatory context: It was mentioned that 30% of the REACH regulation dossiers that so far had been checked, included read-across evaluations. Respective case studies of risk assessment made by ECHA are published on their homepage. Innovative methods developed within the SEURAT-1 Research Initiative could be directly linked into further expansions of read-across evaluations. To target this, a SEURAT-1 meeting at ECHA would be helpful.

Subsequent to the plenary discussion, Catherine Mahony (Cosmetics Europe) gave the meeting participants her take home message, saying that it was worth celebrating the significant progress of projects in meeting milestones and deliverables after the first year and the evolution of cross-cluster working groups. She stressed that it is necessary to engage with a broader scientific community. She finalised by a warning: ‘Caution! I have never seen a problem which, when carefully examined, failed to become more complicated …’.

Derek Knight (acting as a co-chair of the Scientific Expert Panel) concluded the meeting by giving the awards to the selected three best posters. The awards were sponsored by Cosmetics Europe, and provided the possibility for three young scientists to attend a scientific conference of their own choice (with an economical contribution of 500 euro). Derek Knight explained that all posters had been of high standard, and additional criteria besides the scientific interest of the work applied in the winner selection process had been: favourable to initiatives interacting with several SEURAT-1 projects and leading role of a young scientist. The three winners were:

⇒ Francesca Pistollato (JRC): Standardisation of pluripotent stem cell cultures for toxicity testing
⇒ Anja Wilmes (Innsbruck Medical University): Application of the xCELLigence system for monitoring vectorial transport and toxicity of renal epithelial cells
⇒ Mark Nelms (Liverpool John Moores University): Strategies to form chemical categories from Adverse Outcome Pathways

Maurice Whelan (JRC) closed the meeting.

4.9.3 Young Scientist Poster Award

In total, 30 posters covering diverse research activities in the different projects of the SEURAT-1 Research Initiative, were presented at the Annual Meeting (see above). The e-versions of
the posters, as well as the abstracts, are published on the SEURAT-1 public website (www.seurat-1.eu). The poster award committee selected the three best posters, and the awardees present their work in the following extended abstracts (in alphabetical order).

4.9.3.1 Strategies to Form Chemical Categories from Adverse Outcome Pathways

Mark D. Nelms, Steven J. Enoch, Elena Fioravanzo, Judith C. Madden, Thorsten Meinl, Andrea-N. Richarz, Christof H. Schwab, Andrew P. Worth, Chihae Yang, Mark T.D. Cronin

Introduction

With the introduction of the 7th amendment to the Cosmetic Directive (Anonymous, 2003) there has been an increased need to find alternatives to traditional animal testing for cosmetic ingredients. Category formation and read-across are increasingly being seen as in silico solutions for the prediction of toxicological endpoints in risk assessment (OECD, 2007; ECHA, 2010; Spielmann et al., 2011). A chemical category is defined as ‘a group of chemicals whose physico-chemical and toxicological properties are likely to be similar or follow a regular pattern’ (OECD, 2007). A prerequisite for category formation, and thus read-across predictions, is the definition of chemical similarity. Two methods by which chemical similarity can be defined are using structural or mechanistic features. Both of these methods have been used for the formation of categories for a variety of endpoints, including skin sensitisation, respiratory sensitisation and teratogenicity (Enoch et al., 2009; 2010; Aptula et al., 2006; Roberts et al., 2006; 2008; Schultz et al., 2009). One key mechanistic method to group compounds, relevant to the prediction of human organ level toxicity, is the use of reactive fragments (which, when related to physico-chemical or other properties, become chemotypes) associated with known mechanisms of toxicity. Previous research has used the presence of reactive fragments in a molecule as a definition of similarity in order to produce categories relating to a compound’s ability to bind to DNA or proteins (Enoch et al., 2011a; b).

As toxicology moves towards the use of Adverse Outcome Pathways (AOPs), there is an opportunity to link AOPs to categories of chemicals. The key here is the Molecular Initiating Event (MIE), the physico/chemical process that instigates an AOP. It can be associated with well-defined fragments, or chemotypes, that go beyond the classical definition of structural alerts to include physico-chemical properties. The aim of this study was the development of a workflow for category formation based around chemotypes, using a novel platform (KNIME, www.knime.org), in order to predict organ level toxicity as part of the COSMOS project. The workflow was based, at least in part, on the presence of (reactive) fragments associated with toxicity.
Method

Chemotypes were coded computationally either as SMARTS (www.daylight.com) or CSRML (Molecular Networks GmbH). These are computational languages used to describe patterns and properties within a chemical compound that allow searches for specific substructures to be undertaken. The chemotypes are at the core of the workflow to group compounds. The conceptual workflow (as shown in Figure 4.66) was translated into a computational tool using the KNIME software package. KNIME is an open access platform that enables integration of various programs into a transparent format that can be adapted according to the users’ requirements.

Figure 4.66 A schematic representation of the major steps within the workflow that were transferred into KNIME. There are two ways in which the workflow can be utilised to produce categories; using either a chemotype, or a complete structure, as the target compound.

Workflow

Figure 4.66 provides a schematic overview of the strategy to form chemical categories; an example KNIME workflow is shown in Figure 4.67. The workflow can be summarised into various steps as follows.

Step 1 – The structure of the target compound is entered into the workflow and chemotypes associated with known MIEs are identified.

If a chemotype is the input structure start at step 2.

Step 2 – The COSMOS database, containing structures and information on cosmetics ingredients, and/or external toxicological databases are searched for chemical analogues that possess the chemotype of interest.

Step 3 – Any analogues identified, along with their related toxicological data, are retrieved from the databases and used to populate the category.

Step 4 – Toxicity data derived from the resultant category can subsequently be used to facilitate read-across and other in silico predictions of toxicity.

The main advantage to this approach is in applying the knowledge of MIEs, for specific adverse outcomes, to the formation of relevant categories. A limitation of this approach, however, is centred on the availability of reliable toxicity data. In addition, knowledge of the relevant mechanism(s) of action and their MIE(s) are vital.
Associating Chemotypes with Adverse Outcome Pathways (AOPs)

The Adverse Outcome Pathway approach has been promoted to solve the problem of identifying and verifying the mechanisms of action of toxicity and the structural fragments associated with these mechanisms i.e. the chemotypes (Ankley et al., 2010). AOPs record information relating to the perturbation of biochemical pathways which may result in a biologically adverse effect, commencing with the MIE, as shown by Figure 4.68. This knowledge, of associating the MIE(s) to specific adverse outcomes, can consequently be used in the production of endpoint specific categories, supported by data from in vitro / in vivo studies. Due to the identifiable chemistry associated to the MIE it provides a means of classifying the ‘domain’ of the AOP and hence defining chemical space of the AOP.

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**Figure 4.67 Representation of the workflow within KNIME.**

**Figure 4.68 Summary of the steps within an Adverse Outcome Pathway.**
Conclusions

A strategy for category formation, for a multitude of endpoints relevant to cosmetics, based upon knowledge about various MIEs for a variety of adverse outcomes, has been developed. This workflow has been implemented in KNIME as a computational tool to make in silico predictions of chronic toxicity. The COSMOS, and other external, databases can be searched via this KNIME workflow and toxicological data can be retrieved.

References


4.9.3.2 **Standardisation of Pluripotent Stem Cell Cultures for Toxicity Testing**

*Francesca Pistollato*

**Introduction**

Pluripotent stem cell (PSC) lines offer a unique opportunity to derive various human cell types which can be exploited for a more efficient selection of drug candidates as well as mechanistically-oriented safety evaluations of chemicals. However, the demonstration of the reliability and relevance of any toxicity test is mandatory if the tests should be used for regulatory purposes. The peculiar nature of PSCs requires the implementation of additional quality controls in order to reduce intra- and inter-laboratory variability of toxicity tests as well as to define genotypic, phenotypic and functional characteristics of the target cells under investigation, ensuring that the differentiated cell culture is ‘fit for purpose’ and can provide the answer for a particular toxicological question. Therefore, standardisation of undifferentiated cell culture methods, as well as a close monitoring of the differentiation process, will be crucial for a successful stem cell based toxicity testing. One of our main objectives in the SCR&Tox project is the development of quality control (QC) standards that can be applied in routine PSC-based toxicity testing.

**Approach**

In order to define QC standards, we performed an extensive review of currently proposed QCs for PSCs in the scientific literature (*Pistollato et al., 2012*). Hereby, we focussed on the assessment of pluripotency in cell cultures, as well as on the differentiation into five cell lineages which are of interest for the project. In a second step, we have analysed the QC...
standards that are currently applied in selected laboratories participating in the SEURAT-1 Research Initiative by using a prepared questionnaire. This questionnaire is envisaged to develop a minimum set of standards that will be applied to cellular models that are used in the consortium. The submission of data of the different experimental groups to ToxBank is, therefore, of crucial importance to develop such standards. The application of previously agreed QC's will support the data interpretation obtained by using identical chemicals, but various cellular models.

The SCR&Tox partner ‘Joint Research Centre’ has selected a panel of the most critical QC’s which are currently applied on in-house available human induced pluripotent stem cell (hiPSC) lines and preliminary thresholds to be used for the characterization of undifferentiated PSCs and their differentiated neuronal derivatives have been defined.

3. Results and Discussion

In-House Quality Control Analyses for the characterization of IMR90-iPSCs. Undifferentiated IMR90-iPS cells (kindly provided by I-Stem) appeared round in shape, with large nucleoli and not abundant cytoplasm; the colonies were flat and tightly-packed (undifferentiated morphology in more than 85-90% of the colonies; Figure 4.69A) and more than 80% of the colonies were alkaline phosphatase positive (Figure 4.69B). Staining of undifferentiated IMR90-iPS cells for Oct4, SSEA3 and Tra1-60 (not shown) showed that more than 90% of the colonies were Oct4⁺ and Tra1-60⁺ and almost 80% of the colonies were SSEA3⁺ (Figure 4.69C, D). Flow cytometric analyses of SSEA4 and SSEA1 (CD15) showed that more than 85% of the undifferentiated cells resulted to be SSEA4⁺/SSEA1⁻ (Figure 4.69E) with these results were reproducible over passages. To assess stem cell pluripotency, we used the common approach based on ‘spontaneous’ embryoid bodies (EBs) formation, which can form the three germ layers. Analyses of some germ layer specific genes indicated a highly significant increase of endoderm (AFP, KRT18), ectoderm (Nestin, Sox1 and Pax6) and mesoderm (NPPA and Brachyury-T) related gene expression (Figure 4.69F).
Figure 4.69 In-House Quality Control Analyses for the characterization IMR90-iPSCs. (A) Representative phase-bright image of undifferentiated colony; (B) representative image of AP stained colonies; (C, D) Representative immunocytochemical images of (C) Oct4 and (D) SSEA3. (E) Representative dot plot images of SSEA1 and SSEA4 staining. (F) Bar graph reporting qPCR analyses of AFP, KRT18, Nestin, Sox1, Pax6, NPPA and Brachyury-T, normalized to B-actin and GAPDH and then calibrated to their own undifferentiated control (day 0) (ΔΔCt method), mean of 5 independent analyses ±S.E.M.

In-House Quality Control Analyses for the characterization of IMR90-iPSCs-derived Neuronal cells. IMR90-iPSCs have been differentiated toward post-mitotic neurons for 28 days. By plating 1 EB/well in laminin-coated 96-well plates, we found that about 50-60% of plated EBs gave rise to rosette-like structures (neuroectodermal structures; Figure 4.70A, B). Importantly, within these structures, nestin+ cells were highly present and β-III-Tubulin+ neuronal precursors and immature neurons were mainly localized at the periphery of the rosettes (Figure 4.70C). Further differentiation (28 days) resulted in an increase of neuronal related markers such as NF200+, β-III-Tubulin+ and MAP2+ cells (Figure 4.70D, E), together with increase in GFAP+ astroglial like cells (Figure 4.70E). In order to verify the functionality of neuronal like cell cultures, we assessed the generation of extracellular electrical activity by using multi electrode arrays. Analyses indicate that the differentiated neurons generated action potentials, with a mean firing rate of 112 spykes/min (Figure 4.70F).
In-House Quality Control Analyses for the characterization of IMR90-iPSCs-derived Neuronal cells. (A, B) Representative phase-bright images of IMR90-derived EBs at day 1 (A) and of rosette-like structures at day 7 (B). (C, D) Representative immunocytochemical images of β-III Tubulin (Tuj1) and Nestin (rosettes at day 7, C) and NF200 (neuronal cells at day 28, D). (E) Bar graph reporting mean average intensity of neuronal related markers. Mean ± S.E.M. of 5 independent analyses. (F) Representative image of the mean firing rate (i.e. number of spikes/min).

Conclusions

We believe that, for the success of the SEURAT-1 Research Initiative, it is of high importance to agree and harmonize QC standards for stem cell derived cellular models within the different experimental groups. The application of a minimum set of standards will support the data interpretation of in vitro toxicity testing based on these cell models. Such standards can also serve as a basis for the development of a guidance document using these sophisticated stem cell cultures as test systems in routine toxicity testing.

Additionally, our neuronal differentiation protocol drives the differentiation of mature, functional, post-mitotic neuronal cell derivatives. We believe that the neuronal differentiation protocol adopted here, together with the provided QC analyses, might be exploited for designing repeated dose neurotoxicity tests.

References

4.9.3.3 Application of the xCELLigence system for monitoring vectorial transport and toxicity of renal epithelial cells

Anja Wilmes, Alexander Seiler, Alice Limonciel, Lydia Aschauer, Manfred Watzele, Paul Jennings

Introduction
Technological developments are driving in vitro methods towards integrated ‘-omic’ strategies. However, there is still an overreliance on classical viability assays for the planning phase of experiments that include identification of appropriate doses and exposure times. Classical viability assays are not readily suited for the investigation of subtle alterations in cell function and most require termination of the experiment (Limonciel et al., 2011). It is well established that barrier function is an extremely sensitive endpoint for measuring epithelial monolayer integrity. Altered barrier function usually precedes cell toxicity; however, it is technically difficult to measure and requires cells to be cultured on microporous supports. As a consequence of vectorial transport of water and solutes transporting epithelial cells form macro structures, termed domes, when cultured on solid supports. These domes represent an area where the monolayer has lifted from the dish due to an osmotic flux of water following the transported solutes. Here we investigated the applicability of impedance measurements, utilising the xCELLigence system, as a non-invasive tool for monitoring epithelial monolayer formation and barrier function.

Approach
Proximal tubular cells are highly susceptible to xenobiotic exposure due to their high transport capacity and high phase I and phase II metabolism. Here, we utilised the human renal proximal tubular epithelial cell line RPTEC/TERT1 (Evercyte GmbH, Vienna, Austria). These cells were immortalised by the introduction of the catalytic subunit of human telomerase (Weiser et al., 2008). They exhibit many properties of proximal tubule cells including the formation of a stable transepithelial electrical resistance (TEER), due to the expression of tight junction proteins, including occludin and proximal tubule specific claudins.

RPTEC/TERT1 cells were cultured on xCELLigence impedance sensor tissue plates. After a maturation and stabilization phase, the cells were treated with three different nephrotoxins at 7 different concentrations and their impedance was monitored over time.

Results
Initial experiments using different seeding densities showed that RPTEC/TERT1 cells grew
well on xCELLigence impedance sensor tissue plates. Cell Index (CI) values showed a unique impedance profile that could be linked to an attachment phase, a proliferation phase, a maturation phase, and a stabilization phase. The final oscillating stabilisation phase is due to the dynamic dome formation of these cells (Figure 4.71). In response to three different nephrotoxins, the impedance values initially increased due to dome collapse, followed by a decrease of impedance due to cell death. Each compound gave a distinct time response in the altered cell index.

**Figure 4.71** Image of Dome formation on xCELLigence E-Plates recorded on a Cellavista Imaging System and xCELLigence profiles of RPTEC/TERT1 on xCELLigence E-Plates (© Roche Diagnostics).

**Conclusions**

Understanding the biological perturbations brought about by exogenous chemicals will be key to the development of predictive in vitro strategies. To achieve this goal high content -omic technologies will be indispensable. However, such `-omic` experiments are complex and time-consuming. Thus, only a limited number of samples can be analysed. Choosing the optimal concentration of a compound, as well as the optimal exposure time is, therefore, critical for the meaningful design of these experiments. The only way this can be achieved is by correct deployment of preliminary experiments that can be run at much higher through puts. The more sensitive the endpoints employed in these preliminary dose-range finding experiments, the better the chance of success in the `-omic` phase. The xCELLigence system provides an extremely sensitive and non-invasive method to monitor alterations on renal epithelial function and cytotoxicity. It allows not only monitoring various concentrations of a compound, but also provides extremely high temporal resolution. Therefore, it could serve as an excellent tool for monitoring effects in long term repeat dose regimes and be very useful in identifying compound-specific optimal conditions for subsequent `-omics` experiments.
References


4.10 Cross-Cluster Cooperation

4.10.1 The Model of Cross-Cluster Working Groups

The COACH Team

Working groups were created to facilitate the cross-cluster cooperation between projects and people. The overall motivation for establishing these cross-cluster working groups is (i) stimulate project interactions, (ii) to assist the linkage of deliverables from different projects, and (iii) to capture the knowledge spread out over more than 70 partners of the SEURAT-1 Research Initiative. The challenge was to encourage collaborations not foreseen in the lists of deliverables of the individual projects and to find a way to reach further with the SEURAT-1 Research Initiative. It was therefore agreed by the Scientific Expert Panel of the SEURAT-1 Research Initiative that a working group should have two aspects to its profile, one Operational aspect to deal with specific research questions and problems originating from project activities finding common solutions on cluster level, and a Think Tank aspect to encourage creativity and capture external expert views aiming on achieving a large and multidisciplinary prospective.

The first two SEURAT-1 working groups were established already at the first annual meeting. Focussing on the Gold Compound Selection and Data Analysis to assist Toxbank in their tasks to service the cluster, these two working groups were indisputable of interest to all the projects. During the first year while all activities of the SEURAT-1 Research Initiative were started up, it was further discussed which other items would be of such cross-cluster concern, and COACH started to identify core-topics, i.e. ‘horizontal’ cross-cluster activities that would further reinforce the ‘vertical’ project pillars (Figure 4.72).

Figure 4.72 Working groups as horizontal elements to intensify cross-cluster cooperation between the research projects of the SEURAT-1 Research Initiative.
At the second Annual Meeting four additional working groups were created (focussing on stem cells, biokinetics, Mode of Action and safety assessment) based on the break-out sessions organised at the meeting (see chapter 4.9.2). Those were all mirroring identified core-topics and when planning the break-out sessions, multi-project participation was encouraged. The outcomes of the initial discussions have been captured later in this chapter.

The Scientific Expert Panel has discussed and agreed on the Terms of Reference for the SEURAT-1 working groups. It was stated that the work would be carried out on a voluntary basis, and therefore the working groups themselves would set up yearly objectives. The Scientific Expert Panel would have the possibility though to make recommendations or comments taking into consideration the cluster level objectives. To ensure the cross-cluster characteristics of a working group, it was agreed that each working group would have two co-leaders originating from different projects. Working group participation would be open to any project partner, Cosmetics Europe member supported by their Task Force that signed the unilateral declaration of the SEURAT-1 confidential agreement, or Scientific Expert Panel member. External experts can be invited to the think tank activities and related more loosely to a working group because of respect to confidentiality agreements. Coordinators should try to identify key contributors within their project and encourage their participation to relevant working groups. The Annual Meeting at the cluster level will include reports back from the work carried out by the working groups.

COACH supports working group activities by making available dedicated workspaces on the cluster internal website, where all working group documents should be kept also to encourage other partners to inform themselves on working group activities. It is also possible to put forward queries within the frame of the workspace to be followed up by the co-leaders or in discussion with the working group. All partners can subscribe to be a member of a working group via the website. In addition COACH can assist working groups to organise workshops and meetings.

The fundamental idea of the working groups was to add a further horizontal level that will be populated by members from the SEURAT-1 research projects (besides ToxBank and COACH as horizontal orientated service projects, see Figure 4.72) and in the first strategic review of the cluster carried out by COACH, additional core-topics, not so far covered by a working group, will be identified. Based on this analysis and on the interest from the SEURAT-1 partners, it might be agreed to create additional working groups. Core-topics might also be dealt with in dedicated workshops, or re-considered at a later stage depending on other horizontal activities. There must be a critical limit for how many working groups and cluster level activities can be carried out in parallel to the fulfilment of project deliverables, and partners need to be motivated by the added value that might be brought back into the own project. Therefore COACH is planning to carry out annual strategic reviews, to evaluate horizontal activities and expected outcome towards the cluster objectives and by this assist coordinators and partners in setting their own priorities.
In an attempt to exploit the cluster skills and knowledge even further a third dimension could be identified by combining working groups to discuss cross cluster issues. The thinking here is that knowledge has been pooled together in the working groups focusing on the core-topics, but to go one step further, an intelligent combination of the pooled knowledge will give a possibility to tackle additional and even more complex problems with an emphasis on multidisciplinarity work towards cluster objectives.

A SEURAT-1 roadmap will be drafted in the context of the first strategic review to further understand how project main milestones and working group outcome will feed into milestones identified on cluster level to achieve the cluster objectives. The roadmap will then be reviewed and updated on a yearly basis. All projects will individually or in collaboration contribute to the first SEURAT-1 objective, which is to develop highly innovative tools and methodology that can ultimately support regulatory safety assessment. The projects will also through working groups or other coordinated cluster activities contribute to demonstrate the proof-of-concept at multiple levels, from theory to application. The identified proof-of-concepts will then be regarded as cluster milestones, to which projects and working groups will feed in. COACH based on horizontal activities and supported by the Scientific Expert Panel will assist in reaching the remaining objectives.

Table 4.8 provides an overview about the existing working groups of the SEURAT-1 Research Initiative including short descriptions (more detailed working group reports are given in the following chapters). This year will be the first where the capacity of the working groups will be fully exploited, and in the third SEURAT-1 Annual Report the progress of this work will be described.

**Table 4.8 SEURAT-1 working groups in 2012.**

<table>
<thead>
<tr>
<th>Working Group</th>
<th>Co-leaders</th>
<th>WG Description</th>
</tr>
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<tbody>
<tr>
<td>Gold Compound</td>
<td>Jeff Wiseman (ToxBank)</td>
<td>The goal for the Gold Compounds Working Group is to achieve consensus across the SEURAT-1 Research Initiative on the criteria for selecting, accepting and using test substances in the development of alternative testing methods for repeated dose systemic toxicity. Cross-project members and additional external experts collaborate on the discussion of compound selection, mechanisms and assays. A criterion for the compound selection is a preference for previously well-studied compounds for which there is a good understanding of Mode of Action.</td>
</tr>
<tr>
<td></td>
<td>Paul Jennings (DETECTIVE)</td>
<td></td>
</tr>
<tr>
<td>Data Analysis</td>
<td>Glenn Myatt (ToxBank)</td>
<td>The Data Analysis Working Group discusses, on an ongoing basis, best practices, standards and common approaches for program data management and analysis including topics such as vocabularies, protocols, ontologies, statistical analysis, and integrated data analysis. The group is also developing ideas and new approaches to data analysis required by emerging research activities carried out under the programme. The DAWG would also be suitable to host the discussions on the choice of biomarkers and approaches to the processing and analysis of associated &quot;omics&quot; data.</td>
</tr>
<tr>
<td></td>
<td>Annette Kopp-Schneider (DETECTIVE)</td>
<td></td>
</tr>
<tr>
<td>Mode of Action</td>
<td>Steven Enoch (COSMOS)</td>
<td>The Mode of Action (MoA) Working Group will assist in achieving the SEURAT-1 objective to formulate and implement a research strategy based on generating and applying knowledge of MoA. The MoA Working Group should identify known Modes of Action to support the data analysis and outcome from different projects. It is suggested to use the Adverse Outcome Pathway (AOP) framework approach as a practical tool to organise MoA information and capture inter-relations in the cell by means of ‘-omics’ and in vitro data including dose dependencies. A special focus is made trying to link Molecular Initial Events to possible adverse outcomes.</td>
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</tr>
<tr>
<td>Biokinetics</td>
<td>José Zaldivar (COSMOS)</td>
<td>The Biokinetics Working Group provides support to cluster activities in the paradigm shift from pure experimental approaches to a guided model based approach. The Working Group will assist SEURAT-1 projects, not having the expertise available in COSMOS, to design in vitro and bioreactor models and experiments applied to those. To enable in vitro to in vivo extrapolation, the partners need to provide the working group with concentration measurements and effects data from the in vitro experiments. The efforts of the working group are giving strong support to achieve the SEURAT-1 objective to develop highly innovative tools and methodology that can ultimately support regulatory safety assessment.</td>
</tr>
<tr>
<td>Stem Cells</td>
<td>Christian Pinset (SCR&amp;Tox)</td>
<td>The aim of the Stem Cells Working Group is to standardise quality control issues of the cells used in between the different partners and projects. Three cross-consortia cell model subgroups are identified: PSC lines (DETECTIVE, SCR&amp;Tox), EBs (DETECTIVE, SCR&amp;Tox) and Differentiated cell lines (HeMiBio, DETECTIVE, SCR&amp;Tox). The Stem Cell Working Group with support from its subgroups will make it possible to evaluate the competences and robustness of the cell models used and also to ensure that results from different projects using the same cell models are comparable.</td>
</tr>
<tr>
<td>Safety Assessment</td>
<td>Andrew White (Unilever)</td>
<td>The Safety Assessment Working Group will aim to bridge the gap between non animal toxicity testing and the safety assessment decision making needs. Future safety assessment approaches should based on the comprehensive knowledge of the Modes of Action and pathways leading to adverse effects in humans rather than animal testing. The working group will focus on applying the relevant information derived from the developing predictive systems across the projects to progress pragmatic solutions to address the safety decision needs. The group will examine what approaches are useful to build confidence and understand the uncertainty within a mechanistic framework (for example, biokinetic modelling in combination with dose response analysis of in vitro results). As such the group will act as a facilitator to identify key gaps in current knowledge and data needs for the safety assessment decision, working across the regulatory and science space to ensure their generation e.g. to work with ToxBank to identify negatives that realistically help define adaptive versus adverse effects.</td>
</tr>
</tbody>
</table>
4.10.2 Gold Compounds Working Group: Mechanism-based Selection of Reference Compounds for Toxicity Testing Procedures

Jeffrey Wiseman

4.10.2.1 Introduction

The selection of standard reference compounds is a critical issue in any research programme that involves many research groups from different scientific disciplines and needs to be done according to the overarching goals or strategy of the program. In case of the SEURAT-1 Research Initiative, the strategy and goals were outlined in the first Annual Report (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’.

The core concept about how to select the appropriate reference compounds to meet these goals, as well as detailed descriptions about the selected compounds are given in the project report of ToxBank (see chapter 4.7). In brief, the selection procedure was based on the following basic considerations: (i) Extrapolations from well-studied reference compounds to a broader chemical space should be possible; (ii) promiscuity, i.e. a lack of structural specificity in ligand binding, should be considered; (iii) the reference compounds should have well-known Mode of Actions; (iv) the reference compounds should be appropriate to study repeated dose toxicity.

The selection of reference compounds is key for the success of a Mode of Action based approach and should be based on knowledge of different pathways predicted both from chemical and biological information. The starting point is indeed to select chemicals that are extensively studied, i.e. that are very well characterised with respect to their MoA profiles, and this became the major task of the Gold Compounds Working Group.

4.10.2.2 Gold Compound Selection Team

This compound selection strategy has evolved in consultation across the SEURAT-1 Research Initiative. Each project team, the Scientific Expert Panel, and Cosmetics Europe provided representatives at a kick-off meeting in Cascais, Portugal in February, 2011. An advisory Gold Compound Working Group with 20 members was assembled from the attendees at the Cascais meeting, and an evaluation team of six scientists was assembled from the Scientific Expert Panel, industry, and academic labs to evaluate specific compounds for acceptance. As a matter of process, it was agreed that compounds recommended as standards require unanimous agreement by the evaluation team and will be submitted to the working group for review and comment before being accepted as gold compound standards.

1 - On behalf of the Gold Compounds Working Group
The strategy that emerged from the Cascais meeting was defined with respect to adverse events such as steatosis and cholestasis. The evolution to an MoA-based approach was endorsed by the Scientific Expert Panel and developed in a series of monthly teleconferences with the Gold Compound Working Group starting in early August of 2010. Explicit consideration of repeated dose toxicity was initiated at a meeting of experts organized by COACH in Ispra and a subsequent meeting with the SEURAT-1 cardiotoxicity team in Cologne in November, 2011.

### 4.10.2.3 Compound Summary Table

The following table (Table 4.9) summarizes the MoAs and human adverse events for compound standards. Compound suppliers and product numbers are provided to ensure that all labs are using a common compound source.

#### Table 4.9 Summary information for reference standards.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Target organ</th>
<th>MoA</th>
<th>Adverse event</th>
<th>Source and product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drug Standards for Reactive Compounds</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetaminophen CAS # 103-90-2</td>
<td>Liver</td>
<td>Thiol reagent, oxidizing agent</td>
<td>Necrosis</td>
<td>Sigma Aldrich # A7085</td>
</tr>
<tr>
<td>Doxorubicin CAS # 23214-92-8</td>
<td>Heart</td>
<td>Redox cycling, DNA oxidation</td>
<td>Cellular lesions leading to heart failure</td>
<td>Sigma Aldrich # 44583</td>
</tr>
<tr>
<td>Allyl alcohol CAS # 107-18-6</td>
<td>Liver</td>
<td>Thiol reagent</td>
<td>Fibrosis</td>
<td>Sigma Aldrich # 240532</td>
</tr>
<tr>
<td>Carbon tetrachloride CAS # 56-23-5</td>
<td>Liver</td>
<td>Free radical</td>
<td>Fibrosis, steatosis</td>
<td>Sigma Aldrich # 02671</td>
</tr>
<tr>
<td>Aflatoxin B1 CAS # 1162-65-8</td>
<td>Liver</td>
<td>Lysine reagent</td>
<td>Apoptosis</td>
<td>Sigma Aldrich # A6636</td>
</tr>
<tr>
<td>Chlorpromazine CAS # 50-53-3</td>
<td>Liver</td>
<td>Thiol reagent, oxidizing agent, free radical, lipid binding, ATP synthase inhibition</td>
<td>Cholestasis, hepatitis</td>
<td>Sigma Aldrich # 31679</td>
</tr>
<tr>
<td>Iodoacetamide CAS # 144-48-9</td>
<td>All</td>
<td>Thiol reagent (MoA standard)</td>
<td></td>
<td>Sigma Aldrich # 11149</td>
</tr>
<tr>
<td>DMNQ CAS # 6956-96-3</td>
<td>All</td>
<td>Redox cycling (MoA standard)</td>
<td></td>
<td>Sigma Aldrich # D5439</td>
</tr>
</tbody>
</table>
### Promiscuous Ligands and Receptors

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS #</th>
<th>Organs/Tissues</th>
<th>Mechanism of Action</th>
<th>Source</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium valproate</td>
<td>99-66-1</td>
<td>Liver</td>
<td>Inhibition of multiple pathways, including β-oxidation</td>
<td>Sigma Aldrich</td>
<td>P4543</td>
</tr>
<tr>
<td>Amiodarone</td>
<td>1951-25-3</td>
<td>Liver</td>
<td>Phospholipid binding</td>
<td>Tocris Bioscience</td>
<td>4095</td>
</tr>
<tr>
<td>E 4031</td>
<td>113558-89-7</td>
<td>Heart</td>
<td>hERG channel blocker</td>
<td>Sigma Aldrich</td>
<td>M5060</td>
</tr>
</tbody>
</table>

### MoA Standards for Oxidative Phosphorylation

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS #</th>
<th>Tissues</th>
<th>Mechanism of Action</th>
<th>Source</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rotenone</td>
<td>83-79-4</td>
<td>All</td>
<td>Complex I (electron transport) (MoA standard)</td>
<td>Sigma Aldrich</td>
<td>45656</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>1404-19-9</td>
<td>All</td>
<td>ATP synthase inhibitor (MoA standard)</td>
<td>Tocris Bioscience</td>
<td>4110</td>
</tr>
<tr>
<td>FCCP</td>
<td>370-86-5</td>
<td>All</td>
<td>Proton gradient uncoupler (MoA standard)</td>
<td>Tocris Bioscience</td>
<td>0453</td>
</tr>
</tbody>
</table>

### MoA Standards for Lipid Metabolism

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS #</th>
<th>Tissues</th>
<th>Mechanism of Action</th>
<th>Source</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bosentan</td>
<td>147536-97-8</td>
<td>Liver</td>
<td>BESP inhibition</td>
<td>Sequoia Research Products</td>
<td>SRP02325b</td>
</tr>
<tr>
<td>Dirlotapide</td>
<td>481658-94-0</td>
<td>Liver</td>
<td>MTP inhibition</td>
<td>Pfizer (to be confirmed)</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>54910-89-3</td>
<td>Liver</td>
<td>Phospholipid binding</td>
<td>Sigma Aldrich</td>
<td>34012</td>
</tr>
</tbody>
</table>

### Non-MoA Based Selections

<table>
<thead>
<tr>
<th>Compound</th>
<th>CAS #</th>
<th>Tissues</th>
<th>Mechanism of Action</th>
<th>Source</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methotrexate</td>
<td>59-05-2</td>
<td>All</td>
<td>Anti-metabolite</td>
<td>Sigma Aldrich</td>
<td>M8407</td>
</tr>
<tr>
<td>Carbachol</td>
<td>51-83-2</td>
<td>Heart</td>
<td>Cholinergic agonist</td>
<td>Sigma Aldrich</td>
<td>C4382</td>
</tr>
<tr>
<td>(-)-Isoproterenol</td>
<td>7683-59-2</td>
<td>Heart</td>
<td>Adrenergic agonist</td>
<td>Sigma Aldrich</td>
<td>I6504</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>21829-25-4</td>
<td>Heart</td>
<td>L-type Ca channel blocker</td>
<td>Sigma Aldrich</td>
<td>N7634</td>
</tr>
<tr>
<td>Hygromycin B</td>
<td>31282-04-9</td>
<td>All</td>
<td>Protein synthesis inhibitor (standard for electron microscopy)</td>
<td>Invivogen</td>
<td>ant-hg-10p</td>
</tr>
</tbody>
</table>
The SEURAT-1 Research Initiative is addressing hepatic, cardiac, renal, neuronal, muscle, and skin toxicities. The compound selection strategy to date has been developed only for hepatic and cardiac toxicities, and will be expanded with time to other tissues. Further details are given in the ToxBank project report (see chapter 4.7).

References

4.10.3 Data Analysis Working Group: Prerequisites for Integrated Data Analysis
Glenn J. Myatt, Annette Kopp-Schneider

4.10.3.1 Introduction
The development of strategies for assessing toxicity without the use of animal experiments presents a number of unique challenges from a data analysis perspective. Strategies consist of an integration of in vitro experiments, potentially with subsequent ‘omics’ analyses and in silico modeling. Currently, there is no universally adopted standard for collecting and integrating data from different laboratories, experiments and techniques. This makes access for data analysis difficult and time-consuming and presents problems integrating information from different sources in a meaningful way (Sansone et al., 2012). In vivo data, especially human data, is needed to objectively assess the predictivity of newly developed integrated strategies. In vivo data can only be obtained from historical information, which is often difficult to access. There is also no complete ontology for predictive toxicology methods and results to facilitate integration and analysis at a semantic level (Hardy et al., 2012). Furthermore, approaches to data analysis are divergent and evolving, and research is required to understand how to perform an integrated data analysis within a mode of action framework (Whelan & Schwarz, 2011).
4.10.3.2 Relevance for the SEURAT-1 Research Initiative

SEURAT-1 is a complex, multidisciplinary initiative that has adopted a mode of action framework in developing a replacement for today’s in vivo repeated-dose toxicity testing. Data analysis is core to the effective development of these new approaches; however, the data must be captured in an appropriate manner to support any future analysis. Cluster partners are performing research in many diverse areas such as cell differentiation, cell engineering, biomarker identification, dose response analysis, ‘-omics’ experiments, and chemical analysis and many different types of data are recorded in different formats. It is also desirable to integrate this data with external sources, such as information from the Tox21 project (a related initiative in the USA), to leverage these resources as well as human or animal reference data. How the data was generated should be recorded alongside the experimental design, as well as the original and processed results. There are a number of initiatives that are developing standardized approaches to support the collection and integration of experimental data including ISATAB (Sansone et al., 2012) and the Resource Description Framework (RDF) (Carroll & Klyne, 2004). These and other approaches should be discussed and evaluated across the cluster, and adopted where appropriate. Analysis of data from within the cluster and from external collaborators can be used to support each iteration of experimental design.

A number of data analysis procedures are needed in multiple laboratories within the SEURAT-1 Research Initiative to support specific tasks, including dose response analysis and analysis of transcriptomics data. Establishing and documenting best practices will result in consistently generated endpoints across the cluster. It is also important to start the process of developing best practices for evolving data analysis needs, including the integrated analysis of ‘-omics’ approaches such as metabolomics or proteomics.

Developing an integrated data analysis workflow that encompasses the collection, interpretation, integration with biokinetics data, and data analysis will be critical to achieving the SEURAT-1 research goals. This includes the development of strategies within a mode of action framework. There is also a need to understand how to integrate different ‘-omics’ methods at the same time as systems biology approaches. It is important to discuss and assess different approaches with experimentalists with backgrounds in biology, toxicology and chemistry, along with statisticians and database specialists. These discussions should also address issues such as how to sufficiently power the experiments to draw statistically significant conclusions or how to explain results in terms of the underlying biology and/or chemistry. Methods for accessing the information in a convenient manner to perform and subsequently manipulate, integrate and analyse is another important topic that needs to be addressed and could be solved via web services.
4.10.3.3 Objectives of the ‘Data Analysis Working Group’

The objective of the Data Analysis Working Group (DAWG) is to support the data analysis needs of the cluster, including data collection, integration, analysis, as well as experimental design. It provides a forum to discuss issues or problems within the cluster as well as with other academic and industrial groups. This group will discuss best practices, standards and common approaches including topics such as vocabularies, protocols, ontologies, statistical analysis, and integrated data analysis. The group will also develop ideas and new approaches to data analysis required by emerging research activities carried out under the programme, such as the choice of biomarkers and approaches to the processing and analysis of associated '-omics' data.

4.10.3.4 Summary of activities in the first year

At the start of the programme, the cross-project Data Analysis Working Group open to participation by representatives from all consortia was established. Regular virtual discussions were held between the DAWG group members and invited speakers, as well as two face-to-face meetings that coincided with the SEURAT-1 Annual Meetings. Almost 20 events were held including seven events discussing general data analysis needs, five events discussing best practices and a number of presentations from external collaborators such as the Tox21 project, the European Bioinformatics Institute, the ISA team, and the US Food and Drug Administration.

4.10.3.5 Working plan and integration into the SEURAT-1 Research Initiative

It is planned to continue close cross-cluster cooperation in the area of data analysis through the DAWG via both virtual and face-to-face meetings. This would include the development and documentation of best practices in writing protocols, dose response analysis, biomarker identification, as well as integrated data analysis and systems biology approaches. The DAWG will invite external presentations on complementary data generation and data analysis approaches from international initiatives. The DAWG will also develop documentation of data analysis case studies using public and/or SEURAT-1 generated data. As part of these activities, the DAWG will contribute to the SEURAT-1 glossary and provide input for the summer schools.
References


4.10.4 Mode of Action Working Group: Use of Mode of Action Related to Repeated Dose Systemic Toxicity - A Framework for Capturing Information

Mark T.D. Cronin, Andrea-Nicole Richarz

Note: This chapter summarises discussions and conclusions from a Workshop held on 8 February at the 2nd Annual Meeting of the SEURAT-1 Research Initiative in Lisbon, Portugal. The views and opinions expressed here are those of the authors, however we gratefully acknowledge the contribution of a large number of colleagues at the Workshop itself.

4.10.4.1 Introduction and Challenges in the Field

Modern toxicology will be built around biological pathways. Specifically, normal biochemical pathways will be defined that, when perturbed, will result in an adverse effect. If these pathways are known and their relationship to adverse effects at the organ level uncovered, then it will be possible to build in vitro test systems and computational models describing them. This in turn, will provide a framework for collecting information relating to perturbation of pathways.
The ultimate aim must be to map out all pathways relating to toxicity; this ‘map’ will allow for chemicals associated with such pathways, and hence an adverse event, to be identified. This concept and philosophy is not new to the SEURAT-1 Research Initiative, it is at the heart of the Tox21 initiative (http://www.epa.gov/ncct/Tox21/) and efforts such as incorporating mode of action into the assessment of chemical toxicity (Boobis et al., 2008; Meek et al., 2011).

To clarify these concepts, the following loose definitions are used in this chapter and are defined in the context of Figure 4.73. It should be noted that these are not full or harmonised definitions:

- A **toxicity pathway** is a cellular response pathway that, when sufficiently perturbed, is expected to result in adverse health effects (National Research Council, 2007).

- The **mode of action** (MoA) relates to the events including, and downstream of, the toxicity pathway. These could lead to an adverse effect in an individual (Boobis et al., 2008).

- An **adverse outcome pathway** (AOP) represents existing knowledge concerning the linkage between a molecular initiating event and an adverse outcome at the individual or population levels (Ankley et al., 2010).

The **molecular initiating event** is the initial point of chemical-biological interaction within the organism that results in a cascade of events leading to an adverse outcome (Schultz, 2010).

![Figure 4.73](image)

**Figure 4.73** Representation of the relationships between Toxicity Pathways, Mode of Action, Adverse Outcome Pathways and Source to Outcome Pathways. The black bars represent the breadth of these concepts according to current research effort. The gray bars represent the theoretical extent of the concepts. Effects in bold are relevant to the SEURAT-1 Research Initiative in terms of providing information. This figure is adapted from various sources including presentations made by Dr Kevin Crofton (United States Environmental Protection Agency).
The next few years bring a number of practical and theoretical challenges for toxicology to better integrate a mode of action framework. The need for a concerted effort to collect together this information is paramount, as well as an agreement on how this could be achieved. Adverse outcome pathways are being seen as a practical tool to organise mode of action information. To initiate this process a ‘mapping’ of relevant pathways is required to define the foundations of the framework. This will need toxicologists to contribute their knowledge regarding pathways, into which the relevant information can be filled.

It must be understood from the outset that this is a long-term initiative and should not be viewed as a short-term fix. The relevance of the pathways and subsequent models must be consistent with the endpoints being modelled. As AOPs are documented, given that a process of doing this may be established, the concepts of, and needs for, evaluation and possible validation of pathways will have to be investigated.

There is a need to capture the information within the AOPs. Therefore, at the core of this approach is the requirement for a future-proof IT platform that is expandable, flexible and meets the needs of developers and users. The purpose here is not to create a predictive model, but to build a framework linking exposure to adverse effects. In order to make this successful and provide a suitable legacy, the process for recording the AOP needs to be established as soon as possible. For instance, what are the minimum requirements for recording the AOP and what level of detail is required. Another issue will be the level of ‘completeness’ that will be acceptable in an AOP.

There are a number of practical aspects to the collation of AOPs. It soon becomes apparent that, except for some simple pathways, AOPs will be complex, non-linear and highly interrelated. They will be dependent on the dose applied, lifestage of the species and the time after administration (and whether it is a single dose or continued) will affect the perturbation of the pathway and the adverse effect. In addition, the utilisation of the information from the AOP will need to incorporate information regarding the kinetics associated with the administration of a xenobiotic, specifically the likely in vivo cellular concentration.

As AOPs are compiled into templates, what will become apparent is that we will have a collection of (electronic) templates. As these are developed, the flexibility of the system will have to allow for a round (or rounds) of commenting by experts. A wiki-based system would seem to be ideal to capture information in this manner. Whilst a number of systems for capturing this information are being proposed, e.g. OECD Harmonised Template 201 from the European Commission’s Joint Research Centre (EC JRC) Effectopedia from the International QSAR Foundation (http://www.qsari.org/index.php/software/100-effectopedia) and the Chemical Mode of Action Wiki from the United States Environmental Protection Agency (RS Judson personal communication), no one system has yet emerged. A further challenge for the scientific community is to ensure compatibility of these systems.
4.10.4.2 Relevance for the SEURAT-1 Research Initiative and Objectives of the ‘Mode of Action Working Group’

The mode of action concept is a means to organise the information obtained within the SEURAT-1 Research Initiative. Specifically this will enable the information gained from the SEURAT-1 Research Initiative to be placed into larger efforts to capture adverse outcomes, hence linking and formalising effect from exposure, the chemistry of the substance through to the adverse effect. SEURAT-1 partners will identify modes of action and support this information by capturing inter-relations in the cell by means of '-omics' and in vitro data including dose dependencies. A special emphasis will be to link molecular initiating events to possible adverse outcomes.

There are several clear objectives of the Mode of Action Working Group, these can be considered as internal to the SEURAT-1 Research Initiative, and external in terms promoting the capture of pathways.

Within the SEURAT-1 Research Initiative, the Mode of Action Working Group will:

- Promote the mode of action framework as a means of capturing toxicological information relevant to repeated dose exposure
- Develop the use of adverse outcome pathways to relate the chemistry of the molecular initiating event to the adverse effect
- Define the pathways associated with a specific organ toxicity
- Assist in the documentation of the pathways in a flexible electronic manner
- Identify how information from other working groups, e.g. biokinetics, will contribute to the overall toxicity prediction paradigm developed from the AOP approach

External to SEURAT-1, the Mode of Action Working Group will:

- Incorporate knowledge of pathways from the toxicological and systems biology communities
- Engage stakeholders to ensure a high visibility of the AOP approach
- Integrate efforts with other activities e.g. at the OECD, Tox21 and European Commission’s Joint Research Centre

The aims and objectives of the working group will be met through the cross-project co-ordination of efforts in the SEURAT-1 Research Initiative.
4.10.4.3 Working Plan and Integration into the SEURAT-1 Research Initiative

It is clear that there are a multitude of possible adverse effects, organs and pathways that could be documented. The Mode of Action Working Group will attempt to co-ordinate activity in this area. The priority will be an understanding and documenting the pathways associated with liver toxicity. Liver toxicity is chosen as a priority due to importance across sectors (beyond cosmetics) as well as the wealth of information and different mechanisms and adverse effects. It may be overly simplistic to state, but some pathways are already well established, others are more complex and will be greater challenge to modelling. The AOPs for liver toxicity are obviously closely linked to metabolism and other factors such as detoxification and interactions with other factors such as glutathione levels (hence dose as a factor). In order to achieve the goal of documenting liver toxicity AOPs, the following steps will be required:

- A mapping of the modes of action of liver toxicity.
- Within each established mode of action for liver toxicity, identification of specific pathways that are perturbed to elicit the adverse effect.
- Selection of pathways for each mode of action and recording of the molecular initiating event, key events and adverse effects.
- If possible, transfer of the information into a form representative of an adverse outcome pathway, as defined by the OECD and inclusion in a flexible electronic repository such as Effectopedia.

There are clear starting points for the modes of action relevant to liver toxicity, for instance reactive hepatotoxicity, necrosis, cholestasis, steatosis, granuloma, neoplasm and so on. Within these modes of action, pathways associated with toxicity are known e.g. reactive toxicity associated with acetaminophen. The purpose here is not, in the first instance at least, to provide a complete definition of a pathway (although this is ultimately desirable), but to capture the minimum information to provide confidence in the interpretation of an initiating event and its association with key events and the downstream adverse effect.

Within the assessment of these pathways, some should be based around reactive mechanisms (and hence will tie into issues such as the role of metabolism) and others should be non-reactive pathways e.g. receptor mediated. In order to initiate this process consideration could be given to the compounds undergoing current testing and could be assessed within chemical space using, for example, the inventory of cosmetics compounds being developed by the COSMOS project.

The aims will be met only if there is cross-cluster co-operation within the SEURAT-1 Research Initiative and also outside of the cluster.
4.10.4.4 Conclusions

Replacing testing for chronic, organ level, toxicity elicited by chemicals requires a paradigm shift in thinking, belief and understanding of the traditional role of the 3Rs. To make a success of modern technologies and the spirit of 21st Century toxicology, frameworks are being proposed to bring together information on pathways and their perturbation which may result in an adverse effect. A mode of action framework, which may be formalised into an adverse outcome pathway, is such an approach. Within the SEURAT-1 Research Initiative and the broader global scientific community there is an opportunity to collect AOPs for organ level toxicity, with an emphasis on liver toxicity. This must link into projects to capture the information (e.g. Effectopedia) as well as other projects around the world. The important consideration will be that this is seen as a means of compiling information from disparate sources and commercial sectors into a single coherent platform.

References


4.10.5 Biokinetics Working Group: In vitro to in vivo Extrapolation

José-Manuel Zaldívar-Comenges, Alexandre R.R. Péry, Céline Brochot, Bas J. Blaauuboer

4.10.5.1 Introduction and Challenges in the Field

The need to reduce and eventually replace the use of animals in toxicology testing is driving the development of Integrated Testing Strategies (ITS), which aim to predict human in vivo toxic doses from concentrations that cause effects in vitro, without animal testing. This implies the need to consider toxicokinetics (TK) and toxicodynamics (TD) as important, if not essential, parts in the risk assessment strategy (Adler et al., 2011; Basketter et al., 2012). The characterization of the concentration that produces an effect, whether this is a perturbation to a molecular pathway or an apical toxic endpoint, is necessary at two levels; first for the in vitro experiments, since ‘nominal’ concentrations do not represent the real concentration experienced by the cell (DeJongh et al., 1999; Gülden et al., 2001) because compounds may have completely different kinetic behavior in terms of binding to plastic, proteins or lipids, of evaporation and of reactivity; and, second, in the extrapolation of the dose for human toxicity assessment, since to assess the hazard of a chemical compound, we need to know the true concentration experienced by cells within the target organ from the exposure scenario (Blaauboer, 2001, 2002, 2010; Ouattara et al., 2011).

One possible way to solve both problems, and to be able to compare the same concentration values from in vitro and in vivo experiments, is to use TK and TD models at both levels (Escher et al., 2010). Toxicokinetics is essentially the study of the process by which a substance reaches its target site. Four processes are involved in TK: Absorption (A) is the process of a substance entering the organism; Distribution (D) is the dispersion of substances throughout the fluids and tissues of the organism; Metabolism (M) is the irreversible transformation of substances by the organism; Excretion (E) is the elimination of substances from the organism (ADME). The processes and interactions of an exogenous compound within an organism, including the compound’s effects on processes at the organ, cellular, and molecular levels are referred to as toxicodynamics (TD).

Concerning in vitro experiments, it is possible to construct models comprising the fate of a compound in the cell-based assay, i.e., its partitioning between the plastic wall, serum proteins and lipids and potentially the compound’s dynamics within the cell; combined with a cell growth model and a toxic effects model (Kramer et al., 2012; Zaldívar et al., 2011). These together allow us to model the true concentrations causing perturbations near or in cells given the nominal concentrations applied in a microtiter plate well. An analogous approach in vivo is provided by Physiologically Based Toxicokinetics or Pharmacokinetics models - PBTK or PBPK- (Clewell et al., 2008). A PBTK model consists of a series of mathematical equations...
which, based on the specific physiology of an organism and on the physico-chemical properties of a substance as well as its biotransformation and its affinity for transporters (Gosselet et al., 2009), are able to describe the absorption, distribution, metabolism and elimination (ADME) of the compound within this organism (Andersen, 1981). The simultaneous solution of these equations provides the concentration of the chemical compound and its metabolites over time in the modeled organs and allows for a sound mechanistic description of the kinetics processes including the kinetics of accumulation (Loizou et al., 2008).

In parallel, QSPR (Quantitative Structure-Property Relationship) and QSAR (Quantitative Structure-Activity Relationships) techniques can be employed to estimate model parameters such as the partitioning of a chemical in a microtiter plate well medium as well as its tissue partitioning form its physico-chemical properties. Furthermore, the identification of target tissues, possible metabolites and their activity is another area where in silico approaches can contribute.

QIVIVE (Quantitative in vitro-to in vivo extrapolation) has been defined as the framework(s) that allows linking environmental exposures to a chemical to target tissue concentrations that can be compared with in vitro toxicity test effects (Basketter et al., 2012). This quantitative connection needs a combination of several modelling techniques: QSPR/QSAR, PBPK, cell-based and experimental data on metabolism, transport, binding, etc. Several attempts to perform QIVIVE have appeared in recent literature. The first analyses to assess the applicability of this strategy for the safety evaluation of chemicals were carried out by De Jongh et al., (1999), Gubbels-van Hal et al., (2005), and Forsby and Blaauboer (2007). In a more recent approach, Rotroff et al. (2010) estimated the human oral equivalent doses necessary to produce cellular concentrations equivalent to those causing bioactivity in vitro. A recent review summarising end points considered, compounds analysed and overall accuracy of the extrapolation have been recently published by Punt et al. (2011).

To develop further the application of QIVIVE there are challenges to be addressed. These may be summarized as:

- For the in vitro assays: development of analytical techniques to measure free concentration over time (Kramer, 2010) and the effects of chemical reactivity in the medium and metabolism by the cells (Gülden et al., 2010); to measure concentrations inside the cell; to analyze binding, and mediated transport. Furthermore, in vitro cell dynamics (e.g. growth rate) and cell characterization (e.g. size distribution at G1, S, G2 and M stages) should be carried out for each cell system used (Zaldívar et al., 2012). New in vitro models are also necessary in several areas. Current researches in this field are focused on microfluidic bioreactors for in vitro/in vivo extrapolations of ADME processes (Esch et al., 2011; Prot et al. 2011, Sung et al., 2010). Some improvements in the fabrication of the microfluidic bioreactors are still required to reduce non-specific binding.
and to mimic more accurately the in vivo conditions, in terms of biokinetics and adverse pathways.

The calibration of PBTK models is depending on the availability of information with regard to absorption, distribution, metabolism and excretion processes. First absorption rates have to be estimated relative to all considered routes of administration. In particular, getting information on dermal absorption is still an issue, despite some clear improvements in this domain. The metabolism during absorption should also be assessed, in particular for compounds likely to show an intensive metabolism (Van De Kerkhof et al., 2008). Second, there is still a need of a consensus on the estimation of partition coefficients based on QSAR approaches. Recent efforts can be found in the literature to obtain some harmonised framework (Schmitt, 2008). Third, excretion routes and urinary elimination rate can be roughly predicted from molecular structure and glomerular filtration rates (however, specific transporter systems involved should be considered). Moreover, user friendly, open access databases of physiological parameters would improve their development. PBTK models assume normally homogeneous concentrations in each compartment, new approaches for relevant organs, e.g. the liver, based on spatial and temporal dynamics (3D) could help in understanding toxic effects at organ level and when inhomogeneities should be considered. Finally, some information can still be obtained on humans. Techniques do exist to obtain them (exposure to very low doses with measurements of radio-labeled substances).

For the in silico approaches: Improved correlations to predict in vitro protein, lipid and plastic binding, in vivo tissue partitioning, and in vitro-in vivo possible metabolites and metabolism rates (Kramer, 2010). There is good progress in QSAR modeling to assess the main routes of metabolism, but issues remain: is metabolism detoxifying or toxifying? Which validation criteria should we apply? Can we get reliable quantitative predictions?

4.10.5.2 Relevance for the SEURAT-1 Research Initiative and Objectives of the Biokinetics Working Group

The breakout session on biokinetics issues held on 8 February at the 2nd Annual Meeting of the SEURAT-1 Research Initiative in Lisbon, Portugal, was attended by 15 SEURAT-1 participants, and led to interesting discussions that permitted both to reach a better common understanding and to indicate potential interactions between projects of the SEURAT-1 Research Initiative that could be further substantiated through a dedicated working group.
The cell-based assay model being developed within the COSMOS project (Zaldivar et al., 2011; 2012) may be used to better define experimental conditions and protocols for in vitro repeated dose experiments and to assess the differences with single dose experiments. The PBTK models developed also within the COSMOS project may be used to determine relevant concentrations and their dynamics at the target organ. Both models form the basis of the in vitro to in vivo extrapolation approach.

A similar approach to the cell-based assay model can be extended to the bioreactor experiments in HeMiBio and NOTOX consortiums to be able to compare results from both types of experiments and to select relevant doses.

As a conclusion, biokinetics modelling will be absolutely necessary to rationally design in vitro experiments and develop tools to extrapolate in vivo the results obtained in vitro. However, to reach this goal, relevant concentration measurements and effects data are required. Finally, the implementation of modelling approaches during the first phases of an experimental campaign will be able to contribute to a paradigm shift from a pure trial and error experimental approach to a guided model based approach.

Based on that, the objectives of the Biokinetics Working Group are as follows:

- The development of guidelines to combine modelling, analytical chemistry tools and mechanistic knowledge on the toxicity of substances, to design and optimise treatment protocols for in vitro experiments, to best capture temporal dynamics aspects, to define realistic in vitro exposure conditions related to repeat-dose exposure scenarios, and to be able to define common approaches that will allow data inter-comparison.
- To perform in vitro to in vivo extrapolations for selected compounds for which repeated dose toxicity data are available using the virtual cell-based assay and Physiologically Based Toxicokinetic (PBTK) models. This should be carried out by simulating in vitro repeat dose toxicity experiments and developing the corresponding PBTK models.
- To analyse and discuss bioreactor experiments and how relevant models could be developed based on existing in vitro and in vivo approaches.

4.10.5.3 **Working Plan and Integration into the SEURAT-1 Research Initiative**

There is a win-win situation for the interaction between experimentalists and modellers in the projects of the SEURAT-1 Research Initiative concerning in vitro experimentation and the eventual need to perform a proper in vitro-in vivo extrapolation. For the in vitro systems, the modelling can permit to address which parameters in the systems are important to control
or to modify to obtain a better accuracy of the results. It is also the only way to obtain some quantitative and extrapolable results from the *in vitro* tests. Conversely, modelling *in vitro* systems requires information, in particular concentrations (free ones, in particular) outside and inside the cells, i.e. *in vitro* partition coefficients. The same applies to the *in vivo* kinetics assessment. In the context of the SEURAT-1 Research Initiative, toxicokinetic models can be used to help the design of *in vitro* tests by predicting the expected range of concentrations at target level for usually applied doses. Such predictions, based on mathematical models can easily account for uncertainty on parameters, due for instance, to the use of different cell lines or variability in some measurements. As for metabolism, this is still a key issue and the major source of discrepancies between predicted and actual toxicokinetics.

The Biokinetics Working Group agreed to have a meeting during this year attended by representatives from projects of the SEURAT-1 Research Initiative and invited experts to define a roadmap for the development of QVIVE for repeated dose toxicity testing for cosmetics ingredients.

**References**


4.10.6 Stem Cell Working Group: Characterisation and Standardisation of Stem Cells - An introduction

Glyn Stacey, Lyn Healy, Francesca Pistollato, Christian Pinset and Susanne Bremer-Hoffmann

4.10.6.1 Introduction

This chapter summarises discussions and conclusions from a focus session held on 8 February at the 2nd Annual Meeting of the SEURAT-1 Research Initiative in Lisbon, Portugal. The focus session was attended by 17 participants and started with a brief overview of the SCR&Tox activity. One of the key aims that the SCR&Tox project is pursuing is the development of quality control (QC) standards that can be applied in routine pluripotent stem cell (PSC) based toxicity testing. This will be achieved following a step-wise approach based on: (i) judging the toxicological relevance of data derived from stem cell based toxicity tests, (ii) monitoring crucial culture steps, such as in differentiation protocols that will impact on the reliability of the data, (iii) providing guidance for non stem cell in vitro toxicologists in the use of these sophisticated cellular models. In order to define QC standards some SCR&Tox partners (JRC and NIBSC) prepared a review manuscript on markers and QCs that are currently proposed in the scientific literature (Pistollato et al., 2012). The same partners also prepared and distributed to all SCR&Tox partners a questionnaire on possible exploitable QCs for PSC-based toxicity testing; aim of this questionnaire is to provide an exact overview of currently applied QCs. As soon as harmonization of QCs by creation of common templates for both PSCs and primary cells is reached by participating groups, data will be collected and submitted to ToxBank.

In preparation for the meeting Dr. Glyn Stacey and Dr. Susanne Bremer-Hoffmann have interviewed scientists nominated from four consortia, in particular: from NOTOX: Jan Hengstler and Magnus Ingelman-Sundberg; from HeMiBio: Catherine Verfaille; from SCR&Tox: Christian Pinset and from DETECTIVE: Agapios Sachinidis and Mathieu Vinken. During these tele-interviews several questions have been raised on relevant issues related to PSC general cell culture practice and applied quality standards.

4.10.6.2 Fundamental Aspects of Good Practice in Cell and Tissue Culture

Interviewees and participants to the session agreed that the good cell culture practice (GCCP) described in recent literature (e.g., Coecke et al., 2005) is applicable for PSCs. All agreed on the fundamental importance to confirm (i) cell genetic identity by DNA profiling, (ii) the absence of mycoplasma contamination and (iii) the existence of fully informed consent by the donor of the original tissue. Additionally, the use of antibiotics and antifungals should
be restricted to justified circumstances only (e.g. primary cells isolated from tissues heavily contaminated with microorganisms, selection of recombinant clones), in order to prevent suppressing and masking contaminations which may emerge at later stages. Use of cell lines at high passage levels was considered undesirable due to the increased risk of genetic change. Thus, it was concluded that cells should be obtained at low passage number (i.e. P10-20), and early passage archive or master cryopreserved stocks established from which frozen working stocks can be prepared for routine use.

It was agreed that mycoplasma testing should be performed on newly received cells, on samples from cell banks and on a routine basis for cells in culture in the lab. It has been commented that a possible source of mycoplasma contamination might be the primary fibroblasts used as feeder cells, and these should also be routinely tested before use.

In relation to informed consent, the group considered it was important that donor constraints on the use of any lines derived from their tissues and information on original donor genetic data should be clearly documented and traceable by the supplier of the lines who should also provide key protocols for culture and preservation. Sources of information that could be included in further work of the Stem Cell QC Working Group included the SCR&Tox cell line evaluation procedure and hESSCO guidance published by Franklin et al. (2008).

Partners discussed the advantages and disadvantages of using chromosomally non-diploid cells to develop toxicology assays. It was agreed that at the early stages of elucidating the cell biology of response to toxins, it would be important to aim to work with cells of wild-type characteristics (i.e. diploid) as chromosomal abnormalities might cause atypical responses to tested substances. Nevertheless, it is also possible that once key protocols have been established chromosomally altered cell lines may offer growth and stability advantages that would be useful in high throughput screening.

Certain cell culture and analytical reagents (particularly those of biological origin e.g. ‘knock-out’ serum replacement (KOSR), growth factors (e.g. bFGF), monoclonal antibodies) are prone to significant batch to batch variation. It was concluded that vendors should be expected to provide accurate determination of the biological activity of their products. It may also be necessary for the final user to carry out pre-use qualification of some reagents such as bovine serum. In this regard, ToxBank has key deliverables to provide evaluation of suppliers of cell lines and reagents.

4.10.6.3 Review of Cell Types used by SEURAT-1 Partners

Interviewed experts and session participants reported that they were currently using hiPSC and hESC lines, together with several differentiated progenitors and also primary hepatocytes, hepatocyte cell lines (e.g. HepaRG and HepG2), hepatic satellite cells and human neural
stem cells. Differentiated cell derivatives, in the form of cardiomyocyte-derived cells are available within the SEURAT-1 Research Initiative, supplied within SCR&Tox by partner Cellartis (Cellectis), and in DETECTIVE partners are provided with cardiomyocytes by Cellular Dynamics.

As part of the ongoing work within the SEURAT-1 Research Initiative on quality control and standardisation of cells used in toxicology assays, contributors were nominated to set up a number of specialist working groups to identify key parameters for characterisation and quality control of different cell types as follows:

- Primary hepatocytes
- Undifferentiated PSC cultures
- PSC derived hepatocyte-like cells
- PSC derived cardiomyocyte-like cells
- Skin epithelial cells
- Mesenchymal stem cells
- Neural cells

Key issues for these groups will be to establish acceptance criteria for cells intended for use in toxicology assays and the definition of robust positive and negative controls.

4.10.6.4 Developments of Standards for Cell Markers

Requirements for standards relating to cell markers were extensively discussed. In this regard, SCR&Tox had circulated to partners, templates for markers of undifferentiated cells, differentiated cell derivatives and embryoid body (EB) formation and a number of session participants agreed to contribute to provide further information to complete the circulated questionnaire.

Commonly, elevated SSEA-1 has been referred to as a marker of differentiation in human PSCs, but some session delegates expressed the opinion that this marker was not reliable in this respect. An alternative panel of markers to prove initial spontaneous differentiation was discussed by the group. Amongst these, SSEA-3, Nanog and alkaline phosphatase activity have been considered potential markers, as they are typically rapidly downregulated upon differentiation. Other markers such as Oct4 and Sox2 are often transiently down- and then up-regulated upon ectodermal commitment and may therefore give misleading results.

The group discussed verification of marker/gene expression and agreed that it was important to use at least two different methods (i.e. immunocytochemistry and qPCR) when first characterising a PSC line. However, when sufficient data on expected marker expression
levels are developed it was considered adequate to use just one marker assay which provided a rapid and low cost method. The latter requirements were broadly met by qPCR.

During the session an important debate was opened on the definition and measurement of functional assays to be applied on PSC-derivatives. In general, participants agreed it was important to define, using functional assays ideally, the cellular component at two key points in the establishment of cell-based in vitro assays: (i) in the undifferentiated state (for PSC lines) before committing to a differentiation protocol and (ii) in the differentiated state prior to use in an in vitro toxicology assay. Assays proposed for evaluation of differentiated cells included: (i) for cardiomyocytes, impedance measurements, (ii) for hepatocytes, CYP induction, BSA and urea measurements and (iii) for neuronal cells, MEA analysis for electrical activity. It will be crucial to define appropriate threshold values for these functional assays as acceptance criteria to be used for cells before use in toxicity assays. The selection of a specific functional assay should be strongly directed by the type of toxicity assay that the user will perform. The definition of threshold values would be dependent on the cellular model that will be used as positive or negative control (i.e. definition of reference cellular models). Ideally, a functional assay, in order to be valuable at industrial level, should be simple, rapid, reliable and robust, however, approaches to determine robustness may often need to be assay specific and will be established on a case by case basis. In addition to functional assays, analyses of signalling pathway activation should be conducted to define the phenotypic characteristics of the differentiated cell derivatives, in order to pursue a toxicological mode of action (MoA) framework. In general, when using a specific differentiated cell type it is of pivotal importance to characterize it, providing a good scientific description of the model itself prior to exploiting it for toxicity experiments. Robust positive and negative controls, and possibly reference materials, were considered vital to the quality of research developments and definition of these should form an important part of the work of the Stem Cell QC Working Group.

Exploitability of already existing standards for PSC-based toxicity testing has been discussed. Some generic standards have been described for cell cultures in general in the already mentioned GCCP (Pistilliato et al., 2012) and for human embryonic stem cells by the International Stem Cell Banking Initiative group (ISCBI, 2009). However, definition of appropriate standards for cell preparations used in toxicity assays might potentially be complicated if the target cell in vivo is not known. Partners agreed that PSC-based toxicity assay development and validation will require new supplementary standards. In this regard, reference materials, currently under discussion between SCR&Tox and ToxBank (and with other consortia such as ESNATS; ESNATS, 2012), are needed to demonstrate relevance of the cell model and harmonization in the use of compounds for toxicity testing and should involve other SEURAT-1 partners to promote comparability of results.

The group agreed that training on the use of functional assays and/or cell culturing procedures should be conducted directly by hosting partners through the COACH mobility programme,
while basic courses/lessons on PSC biology, especially for young PhD students working within the SEURAT-1 Research Initiative, might be given during summer schools.

4.10.6.5 Utilising ToxBank Resources

ToxBank representatives described how they planned to store methodologies at two levels: (i) ‘research protocols’ (an accurate scientific description of the method) and (ii) Standard Operating Procedures (formally structured protocols submitted with supporting qualification data). The recently created ToxBank website will be accessible to all SEURAT-1 partners to upload SOPs and research protocols which could prove to be a valuable resource for the cluster. Procedures submitted as SOPs should be accompanied by qualifying data and information on positive and negative controls and signed by the principal investigators of the participating groups. Qualifying data should demonstrate the repeatability of the protocol/assay and have well defined controls.

4.10.6.6 Complex Systems Versus Pure Cell Populations

Participants had different opinions regarding the relative value of purified cell substrate population versus complex heterogeneous systems. Purified cell populations might be preferred, especially when performing transcriptomics and impedance measurements, even though cell response to toxic insults and basic cell biology might significantly vary in purified cell culture conditions compared to heterogeneous systems. There was broad agreement that complex cell mixtures and bioreactor models had significant potential to better mimic physiological tissue systems, however, this would also benefit from a better understanding of cell biology in pure cell systems. The group recognised that there is an increasing range of novel research tools now available (e.g. deep-sequencing, analysis of disease-associated SNPs, epigenetics, miRNAs), which might all have a potentially important impact on the value of assay data. However, currently whilst these are valuable for research purposes, considerably more experience with these techniques will be needed to establish them as qualified QC protocols. Feedback from DETECTIVE research activities will be highly valuable in this particular respect.

4.10.6.7 Conclusion

It has been established that the Stem Cell Working Group should continue to work with SEURAT-1 partners to collate the required inputs for the definition of QC templates, according to the questionnaire previously circulated. In particular, SCR&Tox and DETECTIVE partners will collaborate in the definition of QCs for undifferentiated PSC lines and for markers typically
assessing pluripotency (following EB formation). SCR&Tox, DETECTIVE, HeMiBio and other partners will provide inputs to establish acceptance criteria for the differentiated phenotype of toxicology relevant pluripotent stem cell-derivatives and other cell types for use in toxicology assays. These activities will then form the basis of a guidance document on good practice for the development of toxicology assays using human PSC lines.

References

4.10.7 Safety Assessment Working Group: Challenges and Possibilities of a Mode of Action Approach in the Field of Safety Assessment

Elisabet Berggren, Bob van de Water, Andrew White

4.10.7.1 Introduction

The SEURAT-1 Research Initiative was conceived and initiated to drive forward a major paradigm shift in the chemical safety assessment. While success of the various projects will provide new knowledge, technologies, tools and biomarkers to assess pathway level mechanisms, the understanding and clarity on how these can be integrated and incorporated into novel safety assessments as a proof of principle case study is also needed to help deliver the overall cluster level goal.

1 - On behalf of the Safety Assessment Working Group
To start this process an initial breakout session covering safety risk assessment was undertaken at the 2nd annual meeting and a summary of the discussion is provided in this report. Three initial thought starter presentations were provided to cover aspects of (i) current risk assessment approaches and how a case study using a pathway based approach to help understand the utility of this approach; (ii) the use and value of *in silico* approaches for safety assessment; and (iii) critical considerations on the application of *in vitro* models for risk assessment. These were designed to provide different perspectives on the requirements, opportunities and challenges presented by the adoption of a pathway driven approach for safety assessment.

### 4.10.7.2 Current and Future Risk Assessment Approaches

With regard to the current risk assessment approaches for cosmetics it was noted that this is performed at the level of an ingredient and derives from the need to support human consumer trials and marketing products where a change in ingredient, levels or product type is proposed.

The current safety risk assessment incorporates exposure assessment, hazard identification, hazard characterisation and the integration of the aforementioned components into the final safety assessment. The product type is the key determinant of consumer habits which subsequently dictates the route and extent of applied dose. This in turn determines the nature of the risk assessment through defining the toxicological endpoints of potential concern, and also those critical for the safety assessment. The hazard identification focuses on answering questions related to the intrinsic material hazard, the type & severity of effect, and the relevance to humans. It was noted that for many ingredients, toxicological data already exist, and where possible this existing data is used and incorporated as part of the safety assessment. An initial evaluation to support the safety assessment can be based on *in vitro* data, safe history of use (i.e. substantiation arising from epidemiological data) and human clinical data in a weight of evidence approach. Dependant on structural similarity further support from *in silico* methods such as QSAR or read across to similar chemicals with known toxicological profiles can be utilised. The application of TTC may also play a critical role in any exposure led safety assessment approaches. In relation to systemic toxicity the determination of the highest dose that does not produce an adverse effect (NOAEL) enables a safety assessment to be built on a comparison of the predicted consumer exposure based on accepted product use compared to the identified NOAEL. A margin of safety (MoS) is generally used to define acceptability for product use. The MoS incorporates uncertainty factors relating to inter-individual variability in humans as well as species extrapolation based on the worst case scenario, considering human as the most sensitive species.
The limitations of animal testing such as high dose extrapolation and mechanistic relevance in the current safety assessment have been described numerous times. However, some of the key fundamentals of the current approach need to remain central in the development of alternative approaches. This includes the focus on the safety of the ingredient for the product use case to determine the necessary data to inform the safety decision, and also the flexibility to incorporate and integrate multiple data types. Furthermore in terms of integration of data we should learn from previous approaches where, for example, the use of a test battery approaches have resulted in low specificity & high false positive rates.

An ongoing case study using the assessment of DNA damage through the p53 pathway was presented as an example of a pathway based approach for risk assessment. This example highlighted the incorporation of dose response modeling to quantitatively predict the cellular responses that arise from perturbation of the molecular circuits (toxicity pathways) in cells exposed to low-dose stressors. The generation of well defined in vitro assay data based on key components of the pathway, was used to inform on the p53 pathway response across a large dose range and as input into a developing computational model of the pathway. This approach aims to incorporate the dynamic responses of the system to be studied as a whole, including regulatory feedback mechanisms rather than a series of independent assays. To develop the link between the in vitro hazard data and the consumer risk, the use of in vitro to in vivo extrapolation approaches were highlighted. Analytical measurements of the free concentration of the chemical in the media were undertaken to provide a link between the in vitro assays and the predicted blood plasma level. Product-dependent data on consumer usage in combination with in vitro skin penetration studies and physiologically-based pharmacokinetics (PBPK) were also used to derive relevant concentrations for systemic exposure. In summary, while some of the elements necessary to provide information to link the dose-response curve of a defined pathway to the ingredient concentration in a consumer use scenario, have been identified, further refinement is needed to overcome limitations and assumptions currently incorporated into the approach.

4.10.7.3 Regulatory Experience Assessing Chemicals without using Animal Testing

The use and value of in silico approaches for safety assessment

Hazard assessment for classification purposes is as far as possible based on the combination of available information and expert judgement. In silico methods cover, SAR, QSAR and read across and provide opportunities to help in maximising the use and integration of available data.

Read-across is a data gap filling exercise based on existing test results from closely related chemicals (anallogues) which together form a common group or chemical category and has
shown increasing use in recent years. The level of confidence in read across is dependant on (i) having adequate analogue data, (ii) the level of similarity of the compound to the related structures, and (iii) the category rationale to support the prediction. For example read across for 4 Hydroxylamine salts was premised on the basis that salts were no more toxic than the Hydroxylamine compound and were used for read across to carcinogenicity, acute toxicity, specific target organ toxicity, skin and eye irritation, skin sensitisation, and acute aquatic toxicity. Another example provided was the use of read across to classify 118 Nickel compounds based on data provided for 5 nickel compounds previously subjected to risk assessment and their solubility. The rational for this approach was transparent and focussed on the following components: (i) the nickel ion is responsible for the effects to be assessed; (ii) the concentration of the ion at the site of action is the most important factor determining the toxicity of the compound; and (iii) the bioavailability depends on various characteristics of the individual nickel compounds of which solubility in water is considered as being most important for the release of the nickel ion. Solubility in water was therefore used as an approximation of systemic bioavailability of the nickel ion. The level of solubility was used to determine similarity for read across. Carcinogenicity, mutagenicity, and chronic toxicity by inhalation were assessed separately for soluble and insoluble compounds and determined based on the available data for already assessed soluble and insoluble compounds, respectively. However, when continuous parameters are used to define a rational for read across an important consideration is in determining where on this continuum similarity can be separated. For example the question was debated on how sparingly soluble compounds should be classified to determine relevance for bioavailability of the Nickel ion. In comparison where the data indicated that the endpoints were more dependent on the counter ion such as local irritation and acute toxicity the compounds were assessed on a case by case basis, read across could not be considered due to the lack of similarity to the relevant part of the chemical.

In summary QSAR and read-across approaches were used in the pre-REACH days, even though the rationale was not well documented (no standardised reporting formats). Consequently these approaches were not always consistently used between different evaluations. Computational tools were not available to facilitate grouping and read-across as they are today (e.g. OECD Toolbox, Toxmatch and many others). Now is the time to understand how to best apply these tools in the context of classification of chemicals and start to use them on a regular basis. In vitro evidence has been used in a large extent to support assessment of mutagenicity and carcinogenicity properties of chemicals. It should be carefully explored for which properties in vitro data can be used already now and what is the additional proof/ level of confidence needed to make further animal testing unnecessary.

In vitro models and SEURAT-1 - some critical considerations

Both the SEURAT-1 Research Initiative and other TT21C pathway approaches highlight the
use of human in vitro cellular models to provide a strong mechanistic link for human safety assessment. However, 4 key considerations were highlighted that need to be addressed further. These include the range of in vitro models such as 2D monolayers, 3D organotypic models systems and co-culture systems, and also their provenance, whether they are immortalised cell lines, primary cultures or differentiated stem cells. Each brings their own advantages and limitations and techniques are continually being modified to enhance the relevance to the human situation. However, it remains to be ascertained whether markers identified in one system can be applied in other cell systems. While issues around the metabolic capacity of cellular systems have been identified for a number of years, other changes that could impact the sensitivity of the cells for dose response modelling due to their culturing were highlighted. For example, cell lines can switch their energy consumption to glycolysis rather than mitochondrial oxidative phosphorylation (the Crabtree effect) which then impacts on sensitivity of the system for mitochondrial toxicants. Finally, there is a question on the use of averaged population based responses verses single cell analysis approaches that may enable the identification of subpopulations of cells and a sensitivity distribution.

In terms of the biomarkers there is the question of what is the critical marker for sensitively measuring cell function perturbations. For example, some of the identified toxicity pathways may not be directly adverse but act as surrogates for perturbation e.g. Nrf2 in response to oxidative stress. As such, there is a need to understand the context of an adaptive response from an adverse one and relate early targets to later effects through an understanding of functional endpoints for cell health. It is also important that even when biomarkers have been previously identified, e.g. Kim 1 which has been associated with acute necrosis, fibrosis and inflammation in kidney injury, that from a mode of action perspective the initiating events that drive its change in expression can be identified, i.e. are there multiple mechanisms or a single one. Furthermore, in amongst all the possible initiating events that may occur in the signalling network, how do we ensure that all relevant activation events have been captured?

Another important consideration is the impact of the microenvironment of the cells and thus how to translate the findings from an in vitro cell model to the complex organisation found at the organ level. Several examples were discussed including the activation of stellate cells as a critical event in liver fibrosis and the subsequent perpetuation of the activated phenotype by paracrine signaling from injured hepatocytes. In kidney, vasoconstriction can result in reduced blood flow and therefore low oxygen levels perturbing the homeostatic balance. Also highlighted was a working model of drug-induced liver injury where pre-existing conditions, or altered responses, could tip the normal homeostatic balance of the cell towards a more inflammatory state. Subsequent synergistic effects from inflammatory cytokines such as TNFalpha, may drive a change in the hepatocyte from a mild homeostatic drug perturbation or injury to a severe response leading to necrosis or ultimately liver failure.
A discussion was held on the protection goal of both current and what future safety assessments might accomplish. It was identified that this is dependant on the purpose and therefore the regulatory context that the test system is being set up for. For example, there are differences between the needs for pharmaceuticals and cosmetics based on regulatory requirements on whether the system will be utilized directly for safety assessment or as part of a tiered strategy involving prioritization and subsequent further testing. However while a focus within this group would be on the relevance to the cosmetic industry both the utility of outputs from SEURAT-1 and also from other activities ongoing in the development on novel non animal approaches should be seen in terms of their potential to add value to all current development efforts.

While current safety assessments safeguard consumers, the fact that they utilise extrapolation factors based on dose, species and also human population variation in effect obscure the extent of uncertainty and thus how conservative the assessed protection is. A discussion followed on whether with the current available information for known mechanistic pathways the level of confidence attained using the alternative tools could be determined and benchmarked against current in vivo approaches. It was considered that a definition of what is ‘good enough’ could start to be developed through such an iterative process.

A further question raised was the level of uncertainty/ certainty around unexpected adverse effects. It was generally accepted that while advances are increasing the understanding of adverse processes in relation to human biology, we are still currently at an early stage in relation to relating specific biological responses to exposure from specific chemicals. However it was believed that predictive chemistry could form an important component of refining and reducing the potential scope in the toxicological points of concern with further refinement from selected in vitro assays. This was seen in the context of continual process of optimization as improved models and data are generated.

Progress on the safety assessment requires extrapolation across the different spatial components of in vitro cell systems to the human in vivo system, to ensure that relevant exposure is used to define the risk. As such, the use of PBPK models to determine relevant internal doses was considered essential. It was noted that there is still limited knowledge of skin metabolism and bioavailability through the skin. The use of such models was also considered valuable to provide useful dose ranges for assessment of the in vitro models. It was also noted that the free concentration in the media will be necessary to compare across different cellular models and therefore it would be valuable to understand what parameters influence this concentration.
The impact of cellular ‘rheostats’ was actively debated in the context of mechanistic pathways. Rheostats were described as the ability of pathways to modulate the response to input signals such that it can remain relatively insensitive to low level noise (base threshold), but can then rapidly increase the response beyond a certain dose point. Understanding how both feedback and ultra sensitive switches are integrated and what other systemic or confounding factors impact on this either through priming or dampening the system will provide a clearer understanding of the dynamics of the dose response behaviour of these pathways. It was stated that measuring perturbation is not sufficient but understanding how this is connected to the adverse outcome is a critical component to provide a strong mechanistic basis for delineating adaptive responses from adverse and therefore defining regions of acceptable safety. This was also seen as an important area to benchmark against focussed compounds of known mechanism i.e. those identified from the Gold compound Working Group.

The impact of the cell system, its relevance and under which circumstances it is sufficient for use in relation to the safety risk assessment was addressed during the session. For example clarity of relevance against potentially susceptible target populations was discussed. It was raised that from a precautionary approach if you are looking for safety should you look at the most vulnerable populations or cells from a disturbed (stressed) state, and not just the healthy stem or cellular models. It was also noted that for a number of the cell lines the normal cellular networks may not be fully functional due to their cancer background. Therefore the cell model chosen will depend on the pathways being measured and a necessary step will be in assuring that the composition of the pathway is correct for a given assay. It was postulated that one cell system will be unlikely to recapitulate all relevant modes of action therefore integration across different cell models will become necessary.

Also highlighted was the extrapolation needed to assess long term repeat dose toxicity and the clear mechanistic understanding of both perturbation and recovery needed in assessing this. Differentiated traits of the in vitro model have been shown to decline during long-term culture and therefore may not adequately represent the in vivo system beyond a certain time frame dependant on the cell model. However the use of computational models to assess longer term temporal changes was one option identified that could be utilized to predict whether accumulative effect of short term cellular outputs on a toxicity pathway would result in perturbations that would continue to the point of developing adversity or remain within a homeostatic level of the cell due to repair/adaptation.
During the discussion several questions focussed on the number of biomarkers/assays needed, and what is sufficient. It was important to understand their value across different in vitro systems and also the extent of evaluation necessary. This was summed up in the query on what criteria would be useful to set up a battery of tests to qualify as a test system. It was raised that an important starting point will be the question of what does a positive assay result mean? Consequently the assays required would depend on the context of the safety assessment decision. The numbers should accordingly not be seen as a fixed defined set but sufficiently flexible to address critical points of concern. Therefore the assay or assays should be sufficient to determine the impact of a compound on the dynamics of the pathway and its influence on a mode of action. It should also be seen in the context of a possible integrated computational model rather than a single solution. This enables a fit for purpose assessment of an assay to be made based on its reproducibility and biological relevance and separates the output in relation to adverse response as a discussion on the mechanistically relevant mode of action based on knowledge of the biology and the determination of regions of safety to move beyond the bottleneck of comparison to in vivo animal data.

4.10.7.4 Summary and Outputs

The session provided an opportunity to combine expertise across risk assessors, regulators and experimental scientists to explore the challenges and possibilities emerging from the use of a mode of action approach to safety assessment. While there was a recognition that the process is at an early stage and the knowledge base and the available tools are also still in a rapid phase of development, progress to understand their use in safety assessment could be made in parallel. It was agreed that there was a need for a safety assessment working group to be formed across the SEURAT-1 Research Initiative with the following aims:

1) To develop case studies that will help define the context and therefore needs/purpose from a safety assessments perspective of the alternative test systems and use this to identify key gaps in current knowledge and feedback into the projects the data needs.

2) Through an iterative process of optimisation involving risk assessors, regulators and scientists, build confidence in the practical application of pathway-based approaches for safety assessment linking causal outputs to adverse health effects or regions of acceptable risk.

3) To follow up with the ToxBank Gold Compound Working Group in determining
negatives related to modes of action. Case studies to use the ToxBank gold standard compounds as a focused group of chemicals.

4) Provide a focus to disseminate both learnings from case studies and build understanding of safety assessment across the cluster.

5) Aid in the determination of phenotypic attributes and needed to provide confidence of applicability.
4.11 Training and Outreach

The COACH Team

4.11.1 Training Activities

Training the young scientists and in particular the research fellows involved in SEURAT-1 remains a priority. The cluster level training activities are essential for supporting their integration, transferring them the necessary knowledge and for informing them about the specific needs and constraints in the application domains of the research results.

While the initial activities consisted in conceiving a global training programme harmonising the projects initial individual training plans, the past few months have been very productive to effectively launch the activities foreseen in the programme.

![Figure 4.74 SEURAT-1 training activities, from harmonisation to implementation.](image)

The SEURAT-1 training task force (composed of the projects training work package leaders and COACH team members) was proposed and initiated by COACH during the first year of the research initiative (Figure 4.74). It focused on defining a concept for a cluster level training approach and then to start implementing it by organising the first cluster level summer school. This first training event is described in the paragraphs below.

**SEURAT-1 Summer schools**

The first SEURAT-1 summer school took place from 4 to 8 June, 2012 in Oeiras, near Lisbon and hosted by IBET, the Instituto de Biologia Experimental e Tecnológica. Opened in priority to SEURAT-1 research fellows, the event was further opened to "non-cluster participants" and gathered close to 100 participants. The objectives of this first cluster level summer school were to:
spread the knowledge from the **SEURAT-1** related research areas within and beyond the cluster,

provide an opportunity for the research fellows to meet their colleagues from the other research groups, present and discuss their work and also follow courses given by experts,

create synergies and strengthen the collaboration within the cluster.

The 4 days summer school programme, organised over 5 days, featured a mix of sessions including:

- lectures by renowned scientists,
- presentations by PIs and young researchers,
- practical hands-on workshops,
- poster sessions.

For more concreteness, visits of IBET’s Animal Cell Technology Laboratories, their Pilot and cGMP manufacturing plants were organized.

*Figure 4.75* shows how the programme was designed to allow up to 33 training classes, organised through parallel sessions (hands on sessions were limited to a maximum of 25 participants).

To come up with this programme, COACH stirred the training task force using a bottom up approach. The idea was to pull the cluster participants training wishes and training offers and to then identify a top list of topics.

The training work package leaders then went back to their respective consortia to identify
potential speakers for each session, while COACH liaised with the keynote lecturers and took over the overall organisation and logistics. A detailed programme was put together, identifying the following elements:

- session title,
- lecturer, organisation, project
- short description of the course content
- learning objectives
- duration and logistics details
- expected profile of the participants or pre-requisites

The programme consisted of the sessions listed in Table 4.10.
### Table 4.10 Detailed programme of the SEURAT-1 summer school.

<table>
<thead>
<tr>
<th>Monday 4 June 2012</th>
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<tbody>
<tr>
<td>Welcome to the 1st SEURAT-1 Summer-School</td>
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<tr>
<td>Keynote lecture on «Safety role in the Cosmetic R&amp;D process»</td>
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<tr>
<td>Keynote lecture on «Basics in toxicology»</td>
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<td>Applications of 3D cell cultures in fully controlled bioreactors – special emphasis on liver cells</td>
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<th>Tuesday 5 June 2012</th>
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<tr>
<td>Liver biology and tissue engineering as models of drug metabolism</td>
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<td>Keynote lecture on “Approaches to identification of biomarkers”</td>
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<td>Presentation on in vitro toxicity systems</td>
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<td>Introduction to computational toxicology</td>
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<td>Presentation on functional readouts of toxicity</td>
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<td>Protocol and data management</td>
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<td>Mechanism and compound selection</td>
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<td>Presentation on ‘-omics’ readouts of toxicity</td>
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<td>Hands-on (ToxBank Data Warehouse)</td>
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<td>Repeated hands-on (ToxBank Data Warehouse)</td>
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<td>The potential of genomics technologies enabling ASAT- assuring safety without animal testing</td>
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<th>Wednesday 6 June 2012</th>
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<tr>
<td>Repeat dose toxicity testing and risk assessment</td>
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<td>Use of non-standard methods in regulatory science: challenges &amp; opportunities illustrated by REACH &amp;CLP</td>
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<tr>
<td>Computational modelling of liver toxicity: Role of adverse outcome pathways and prediction of metabolism</td>
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<td>Pluripotent stem cells as source of hepatocytes</td>
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During the same week, a Scientific Expert Panel (SEP) meeting was scheduled at the same location. The aim of embedding the SEP meeting in the Summer School was to create opportunities for exchanges between the scientists involved in SEURAT-1 research and the SEP members and to encourage the SEP members to make talks in some of the summer school sessions.

For creating further opportunities for exchanges among the participants, 3 poster sessions were organised throughout the event. A call for posters was launched at registration opening: each participant was invited to submit a poster on his/her research work within the SEURAT-1 cluster, (especially welcoming posters showing cross-cluster collaborations). In total, 34 posters were presented on this event.

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<th>Thursday 7 June 2012</th>
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<tr>
<td>Current state of the art in genotoxicity testing</td>
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<td>Liver cell types for drug toxicity testing</td>
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<td>Blood clearance function of the liver, with special reference to the liver sinusoidal scavenger endothelial cell</td>
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<td>Introduction to KNIME workflows and how to build models in KNIME</td>
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<tr>
<td>Mechanistic interpretation of ‘omics’ data</td>
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<tr>
<td>Introduction to the threshold of toxicological concern (TTC) concept</td>
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<td>Using literature resources to build biological pathways models for long term toxicity</td>
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<tr>
<td>Understanding and improving primary hepatocyte cultures for systems biology and toxicology</td>
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<tr>
<td>Metabolic fluxes as potential indicators of drug induced effects</td>
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<td>Drug-induced liver injury: Mechanisms, types and biomarkers</td>
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<th>Friday 8 June 2012</th>
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<tr>
<td>Good cell culture practice</td>
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<tr>
<td>Technologies for fabrication of microfluidic bioreactors</td>
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<tr>
<td>Pluripotent stem cells &amp; quality control</td>
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<tr>
<td>Introduction to data governance, databases and chemical space for predictive toxicology</td>
</tr>
</tbody>
</table>
In order to give the priority to SEURAT-1 research fellows the registration was initially only opened for them. In a second step, it was opened to scientists not involved in the cluster so as to spread the knowledge beyond the cluster’s borders.

This first SEURAT-1 summer school was key to support the integration of the young researches so as to increase efficiency as much as possible in their on-going research and development work. The feedback received from the participants was extremely positive and they were very appreciative on the quality of the presentations and the practical organisation. The event was found to be a suitable environment for scientific exchanges and the creation of long-lasting links between groups working in related research domains.

**Hands-on lab training (project level)**

*HeMiBio Winter School:* A winter school entitled “Introduction to Microfabrication Technology for Biology and Medicine” was organised by the HeMiBio partner Dr. Yaakov Nahmias from 16 - 17 January 2012 at the Silberman Institute of Life Sciences of the Hebrew University of Jerusalem, Israel. Microfabrication technology has already changed our local environment, as it is routinely used in everyday products such as cars, music players, televisions and cell phones. Nanotechnology enables an unparalleled control over electrical signals resulting in significant computational, communication, and memory powers. A similar revolution is currently changing the study of biology and the practice of medicine. Microscale patterns, 3D features and microfluidics allow us to screen thousands of conditions, control the cellular microenvironment, and provide innovative tools for the diagnosis and treatment of disease.

This winter school was designed for young investigators who are active in biomedical research. The school introduced the participants to the essentials of microfabrication technology with a series of frontal lectures and hands-on laboratory modules. In particular, the practical and theoretical experience acquired in this course improved communications between biologists and engineers by providing a better understanding of the potential and limitations of microfluidic technology.

**4.11.2 Workshops**

Besides the working group meetings (see chapter 4.10) additional workshops were organised addressing specific aspects of repeated dose systemic toxicity. Participants were experts (both internal and external) in the respective fields, as the intention was to discuss open questions in the field on a high level and provide suggestions for future activities. In principal, the workshops were intended to be used as a starting point for collaborations between cluster projects of the SEURAT-1 Research Initiative and other related international activities.
Workshop on mechanisms underlying repeated dose systemic toxicity

The workshop was held from 14-15 November 2011 at the European Commissions “Joint Research Centre” (JRC) in Ispra, Italy. The workshop was organised by Michael Schwarz and Maurice Whelan, both as representatives of the coordination project COACH of the SEURAT-1 Research Initiative. The 24 participants were experts from the SEURAT-1 Research Initiative as well as invited external experts in the field with complementary fields of knowledge and experience, to enable a focussed and purposeful discussion.

It was the first SEURAT-1 workshop on mechanistic aspects of the Mode-of-Action (MoA) of chemicals causing toxicological response in human trying to focus on repeated dose systemic toxicity. The aim of the workshop was to summarise the multiple mechanisms underlying repeated dose systemic toxicity and to identify representative examples (case studies). Michael Schwarz (University of Tübingen) presented a case where toxicity in one cell type (hepatocytes) is directly connected to reactions in another cell type (stellate cells); repeated dosing of carbon tetrachloride leads to irreversible damage to the liver, while effects of one single high dose (causing cell death) are completely reversible. In most cases where reactive intermediates play a role in toxicity, fibrosis is resulting from hepatocellular necrosis (even though there are examples where this is not so). Therefore, determination of the initial insult may be sufficient to predict the repeat dose outcome. It was concluded that the toxicokinetics must be carefully addressed in in vitro tests, but the questions how to implement repair mechanisms and combined effects from disturbances in different organs in such systems remained open. Jeff Wiseman (Pharmatrope) presented the chemical selection strategy for the SEURAT-1 Research Initiative (reported elsewhere in this book, see chapter 4.10.2 and 4.7.2). The discussion lead to the recommendation that the first set of chemicals should be characterised with one dominant mechanism, and only as a second step it would be advisable to choose chemicals with competing mechanisms based on the understanding of the result from the first set of tested chemicals. Furthermore, it was stressed that the use of biomarkers is the most efficient way to distinguish between different chemical and biological effects.

Cliff Elcombe (CXR Biosciences Ltd) focused in his presentation on chemically induced testicular dysgenesis syndrome, a case from the field of reproductive toxicity. Chemicals can interact with the orphan nuclear receptor SF-1 and, thus, interfere as agonists in several biological pathways. It was concluded that the general strategy for receptor-mediated toxicity would be to separate the different receptors to test and elucidate the different modes-of-action. Once the mode-of-action as well as the interaction between the receptor and ligands (as the molecular initiating event) is known, read-across approaches for predicting the toxicity of so far not tested chemicals may be applicable. However, a chemical can have several modes-of-action and interact as agonist ligands not only with one, but two receptors as presented by Peter-Jürgen Kramer (PJK) in the case of pharmacological agents with high affinity for both the Serotonin 5-HT1A and the Dopamine D3 and D4 receptors. In addition, repeated administration
of low doses revealed clear toxic effects in two target tissues, while the observed acute toxicity of the compounds was very low, highlighting again the importance of the toxicokinetics.

Hennicke Kamp (BASF SE) presented results from in vivo studies focussing on metabolomics, i.e. the chemical pattern at the end of a cascade of reactions. These chemical patterns, which correlate with a physiological status of the organism, can be inversely used to identify the mode-of-action of a certain chemical. Based on this case study, it was questioned whether it would be necessary to understand the complete mechanism first, and then find a system to mimic it, or whether it would rather be possible to screen and build read-across on the biological profile of a substance compared to the one of another. The presented metabolomics examples were based on animal data, but it would be possible to set up an in vitro system to make similar metabolomics profiling. Currently such a system is under development using HepG2 cells.

Ivan Rusyn (University of North-Carolina) focused in his presentation on the problem of interspecies differences with respect to toxicological responses. He concluded in the context of receptor-mediated toxicological responses (e.g. PPARα) that toxicogenomic data and pathway-based approaches have a high potential for better understanding the mechanisms underlying species differences in toxic responses to nuclear receptor agonists. In addition, Kevin Park (University of Liverpool) focussed on intraspecies variability by presenting some lessons learnt from studies regarding drug hypersensitivity and drug-induced liver injury (DILI). The importance of biomarkers for predicting drug-induced toxicity was discussed based on case studies about piperacillin (hypersensitivity) and acetaminophen (DILI). The identification of such biomarkers, however, critical relies on mechanistic understanding about the biochemical processes behind these side effects. Brigitte Landesmann (JRC) presented a conceptual model for liver toxicity covering all aspects from the chemistry of the toxicant, the molecular initiating event of a toxicological mode-of-action to cellular and organ responses. She discussed the model approach in the context of acetaminophen toxicity. In the case of acetaminophen all different toxicologically relevant pathways lead to mitochondrial damage, and thereafter necrosis, apoptosis or steatosis. It was assumed to be useful to define a group of “risk proteins” in a cell to qualitatively predict cell death.

In summary, knowledge coming from investigation of adverse effects of pharmaceuticals was identified as being crucial, as this is a field where experience about toxicity in humans already exists. Timing of different events in cell models, and what can be regarded as acute and repeated dose toxicity was discussed. Other issues identified for further thought were; (i) the relevance of cell models, (ii) how to address intercellular events and (iii) the need of better biokinetic prediction models. It was agreed that a better knowledge of Adverse Outcome Pathways (AOPs) would be the basis for the understanding of the key events possible to observe from in vitro models to enable toxicity predictions of chemicals.

The workshop stimulated the strategic planning of the SEURAT-1 Research Initiative (see
chapter 3) and influenced the programme of the second annual cluster meeting (see chapter 4.9.2) leading to the establishment of a cross-cluster working group focussing on Mode of Action in the field of repeated dose systemic toxicity (see chapter 4.10.4).

**Workshop on paradigm shift anticipation tools for regulatory agencies (written by Sébastien Duprat and Vania Rosas from SCR&Tox)**

We largely describe through different chapters of this book the fundamental changes in conceiving tomorrow’s toxicology, driven by the expected wave of assay validation using high throughput cell based approaches. The generalization of such biotechnological tools is increasingly well understood and exploited by scientists but remains a very obscure concept for regulatory bodies.

Therefore, SCR&Tox initiated in conjunction with its 1st Annual meeting in February 2012 a proactive arena of interaction between SCR&Tox partners and European and national regulatory authorities. Its first occurrence gathered in a workshop a roughly equal number of regulatory body representatives and scientists of the consortium. We covered topics ranging from limitations and opportunities of various iPS reprogramming technologies towards current situation and futures challenges in industrializing iPS assays or learning from cellular assay validation history. Recurrent comments over the advanced status of this technology and the quickness over which we may expect emergence of concrete applications in the field demonstrated how necessary and urgent it was to set up such interaction.

SCR&Tox is planning to extend the reach of the initiated dialogue, either alone or – and that is strongly supported by its executive committee – in conjunction with other European consortia. For this, we will investigate opportunities to graft cell based assays-related topics in existing established meetings of regulatory authorities throughout Europe.

4.11.3 Conferences

Throughout the first year of cluster existence, SEURAT-1 was represented in numerous events by COACH team members. An excerpt of SEURAT-1 representativeness in major international events is shown in Table 4.11.
Table 4.11 Presence of the SEURAT-1 Research Initiative in international conferences and workshops

An overview of the DETECTIVE project (in poster format) was furthermore presented by VUB to the public in August 2011 at 8th World Congress on Alternatives.

COSMOS was presented at the 8th World Congress on Alternatives and Animal Use in the Life Sciences on 21 – 25 August 2011 in Montreal, Canada, and was also represented at the SOT 51st Annual Meeting, on 11 – 15 March 2012 in San Francisco, USA. An overview of the project and results were presented in substantial contributions to the 15th International Workshop on Quantitative Structure-Activity Relationships (QSAR 2012) on 18 – 22 June 2012 in Tallinn, Estonia.

ToxBank presented the approach of data sharing at the cluster level on the 48th Congress of the European Societies of Toxicology (EUROTOX) on 17 – 20 June 2012 in Stockholm, Sweden.
4.11.4 Public Website

The SEURAT-1 website (www.seurat-1.eu, Figure 4.76), online as of September 2011, aims to support dissemination of information about this Research Initiative, its strategy and its results. The website contains the SEURAT-1 Annual Reports, brochures, posters and leaflets, but also scientific publications in the related research domains. It is also a mean to communicate on open jobs at the participating organisations and on cluster projects events and workshops.

In addition, the website contains a searchable directory (who’s who) aiming to present the individuals involved in the cluster and to foster establishing contact with them.

Regularly updated and enriched with information pulled from and pushed by the cluster projects, the SEURAT-1 public website evolves along with the cluster activities to reflect the most recent findings and results.

![SEURAT-1 public website homepage (www.seurat-1.eu).](image)

The SEURAT-1 website targets a large audience ranging from experts, scientists from related research projects, potential users of the knowledge and technologies resulting from this research work, regulatory agencies, policy makers, public funding authorities, as well as the general public. The content is therefore adapted to provide key information of interest for all these target groups and more detailed information for experts, together with links to complementary information sources.

Figure 4.77 shows that the SEURAT-1 public website is an excellent window to showcase the activities and outcomes of the cluster: in 8 months, the website was visited close to 4,500 times, with 13,500 page views.
Peaks of visits can be observed at key milestones: at Annual Report (vol. 1) on-line launch and at the time of the annual meeting.

Interestingly, the SEURAT-1 Research Initiative raises worldwide interest, as shown in Figure 4.78. Most frequent visits are from France, Germany and UK, but the number of visitors from the USA are actually higher than from Italy and Belgium. Japan is ranked seventh, which also strengthens the idea of international interest into SEURAT-1.

Figure 4.77 SEURAT-1 website visitors overview (source: google analytics)

Figure 4.78 SEURAT-1 website visitors location (source: google analytics)
4.11.5 **SEURAT-1 Dissemination Material**

Published at the end of September 2011 on the SEURAT-1 public website, the printed version of the first Annual Report was available a few days later for sending to the various target groups. Over the last 8 months, about 1400 copies were sent by post or distributed at large events, like the EPAA and ECOPA annual conferences. Reflecting the high interest generated by this publication, the webpage where the report can be downloaded counts 850 hits. The COACH office received about 60 individual requests of printed copies via the order form available via the SEURAT-1 public website.

Specific printed material was prepared so as the increase the visibility of the SEURAT-1 Research Initiative at large events. The following documents were distributed:

- a SEURAT-1 Leaflet
- A poster summarising the research strategy of the SEURAT-1 Research Initiative (presented at the 8th World Congress on Alternative testing strategies, *Figure 4.79*)
Additional documents were prepared in view of communicating on **SEURAT-1** to the widest audience:

- A Who’s who booklet, presenting the curriculum vitae of each individual involved in **SEURAT-1**, also available in electronic format on the public website.
- PowerPoint presentations available for all the cluster participants:
  - A full version presenting **SEURAT-1** and including an introduction on each cluster building block
  - A short five-slide version providing an overview of **SEURAT-1**
  - A two-slide presentation that can be included in any presentation from the cluster participants
“It is important that all alternatives to animal testing research, including SEURAT-1, is considered in the framework of creating a longer-term roadmap to ensure that all [...] research efforts are united - and research investments optimised - in order to achieve workable solutions to replace animal testing as soon as possible.”

Bertil Heerink, Director General of Cosmetics Europe, In: Cosmetics Europe press release, published on 8 February 2012.

http://www.cosmeticseurope.eu/news-a-events.html
5.1 Introduction

Taking into account the complexity of the problems to be solved and the broadness of the expertise needed to address the underlying scientific questions, the SEURAT-1 Research Initiative will not be able to finalise the necessary work for full replacement of animal testing in the area of repeated dose systemic toxicity within the next years. Indeed, moving from animal testing to mode-of-action based in vitro assays for improved human safety assessment will require the combined efforts of European and other international activities. The SEURAT-1 Research Initiative is operating in a very dynamic field of research, and a number of related research projects are active in parallel in different parts of the world. This chapter will provide an overview about these parallel running 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 international co-operation as close as possible, over the course of SEURAT-1, and to advance scientific progress in this field of research by using the synergy of a collaborative approach that needs to be developed. This will provide the basis for the identification of gaps of knowledge that needs to be addressed in the future. To start these joint activities, international leading scientists were, and will be, invited to the Annual Meeting of the SEURAT-1 Research Initiative. As reported in chapter 4.9.2, Melvin Anderson from the USA gave a keynote lecture in the second Annual Meeting. He is currently involved in research programs addressing similar areas as the SEURAT-1 Research Initiative. His contribution to the Annual Meeting resulted in a report about the in vitro tools for the 21st risk assessment. This contribution finalises this second Annual Report with the implicit message that the SEURAT-1 Research Initiative has not only started the research work in the various projects, but also initiated the first efforts to set up international collaborations needed to jointly establish the paradigm shift from descriptive to mechanism-based, predictive toxicology.

5.2 Related International Activities

The COACH Team

The following sections are a follow-up of summary reports about ongoing international activities in research areas that are related to the SEURAT-1 Research Initiative. This was started in the first volume of the Annual Report and, together with the updates in this second volume, provides an overview about parallel research activities as a basis for future collaborations.
between SEURAT-1 and other consortia. The descriptions were 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).

5.2.1 European Activities

EU FP7: 7th Framework Programme of the European Union represented by the European Commission

A working document (not legally binding) focussing on future innovative health research was published on 19 April 2012. As outlined in this working document, a new call for proposals entitled “Modelling toxic responses in case studies for predictive human safety assessment” may be published soon under the HEALTH Theme. The main objective of this topic is to exploit the recent advances in computational chemistry and systems biology in case studies, in order to provide the basis for innovative approaches to predictive human safety assessments. Integrated research should be undertaken that:

- Considers modelling transport and interactions from molecular to cellular/organelle levels
- Integrates with in vitro experimentation designed specifically to inform this modelling
- activity
- Couples directly to systems modelling from cellular to organ level
- Takes into account the mechanistic understandings of toxic responses in specific organs; and
- Uses existing and appropriate infrastructure for computation data basing and sharing.

Besides the development of a comprehensive strategy and research concept, the following issues should be addressed either at the theoretical or at the experimental level:

- Identifications of metabolites (and metabolites of metabolites) and their reactivity, through a combination of computational chemistry, in vitro experimentation and enzyme expression profiling.
- Identification of the proteins and potentially other intracellular targets, affected by each metabolite, through computational chemistry and in vitro work.
Identification of the pathways affected by these proteins, through in vitro cell assays and systems biology.

Identification of cell functions affected by these pathways, by defining the boundaries of normal function, and understanding of the physiology and systems biology.

The relationships with the SEURAT-1 Research Initiative are obvious and a close cooperation will be established once such a project receives funding.


IMI: Innovative Medicines Initiative

As already introduced in the first volume of the Annual Report, IMI is Europe’s largest public and private sector collaboration between public authorities, biopharmaceutical companies, patient organisations, universities and other organisations. IMI’s research projects that are selected for funding through open calls for proposals have to follow the four areas (the so-called Four Pillars) of the Strategic Research Agenda: (1) Predictivity of Safety Evaluation, (2) Predictivity of Efficacy Evaluation, (3) Knowledge Management, and (4) Education and Training. Within the 4th Call for proposals that was published in 2011, IMI addresses 7 topics distributed over the following three areas: (i) EU Medical Information System, (ii) Chemistry, Manufacturing and Control and (iii) Technology and Molecular Disease Understanding. The latter comprises the topic “Human Induced Pluripotent Stem (hiPS) Cells for drug discovery and safety assessment”, which is obviously closely related to the field of the SEURAT-1 Research Initiative (i.e., SCR&Tox and HeMiBio). The deadline for full project proposals was 13 March 2012 and the evaluation procedure was expected to be finalised by May 2012. IMI’s 5th Call for proposals (to be published in summer 2012) includes one indicative topic, which is “European lead factory: Joint European compound library and screening centre”.

IMI is currently running an education and training programme on drug safety, called the SafeSciMET programme. Based on a close collaboration between academic institutes throughout Europe and members of the European Federation of Pharmaceutical Industries and Associations (EFPIA), SafeSciMET presents a unique pan-European network, developing and establishing a comprehensive education and training programme in safety sciences for medicine. It involves a two-year programme in total, comprised of different modules covering all topics of modern safety sciences. This includes courses on Cellular Toxicology, Molecular Toxicology, Toxicogenomics and Systems Toxicology and Pharmacogenetics.

eTOX: Integrating bioinformatics and chemoinformatics approaches for the development of expert systems allowing the in silico prediction of toxicities

eTOX is funded by the Innovative Medicines Initiative (IMI) Joint Undertaking that started in 2010 and will run until December 2014. The consortium comprises 25 partners from academia and industry including SME’s. 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 early stages of the drug development pipeline. This will be achieved by jointly storing and exploiting private data from the participating European Federation of Pharmaceutical Industries (EFPIA) and Associations EFPIA companies, as well as publicly available data, and by coordinating the efforts of specialists from EFPIA pharmaceutical companies, relevant SMEs and academic institutions. The proposed strategy includes a synergetic integration of innovative approaches in the following areas:

» Database building 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 of 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 which govern in vivo toxicological problems.
» Computational genomics to afford the inter-species and inter-individual variability that complicate 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.

Hence, eTOX is operating in fields that are related to the SEURAT-1 projects COSMOS and ToxBank.

Project coordinator: Ferran Sanz, Fundació Institut Mar d’ Investigacions Mèdiques, Spain

More information: http://www.etoxproject.eu/
diXa: Data Infrastructure for Chemical Safety

‘Data Infrastructure for Chemical Safety’ (diXa) is funded under the European Commissions 7th Framework Programme. The project started in October 2011 and will run until September 2014. The main objective of the diXa project is to further develop and adopt a robust and sustainable service infrastructure (e.g. data infrastructure and e-science environment) for harbouring multiplexed data sets as produced by past, current and future EU research projects on developing non-animal tests for predicting chemical safety, in linkage with other globally available chemical/toxicological data bases and data bases on molecular data of human disease. diXa focuses on networking activities for building a web-based, openly accessible and sustainable e-infrastructure for capturing toxico-genomic data, and for linking this to available data bases holding chemico/physico/ toxicological information, and to data bases on molecular medicine, thus crossing traditional borders between scientific disciplines and reaching out to other research communities.

To advance data sharing with research communities, diXa ensures clear communication channels with and deliver commonly agreed core service support to the toxico-genomic research community, by providing SOPs for seamless data sharing, and by offering quality assessments and newly developed tools and techniques for data management, all supported by hands-on training. Through its joint research initiative, by using data available from its data infrastructure, diXa will demonstrate the feasibility of its approach by performing cross-platform integrative statistical analyses, and cross-study meta-analyses, to create a systems model for predicting chemical-induced liver injury.

Scientific Coordinator: Jos Kleinjans (University of Maastricht, The Netherlands)

More information: http://www.dixa-fp7.eu/

EUROECOTOX: European Network for Alternative Testing Strategies in Ecotoxicology

EUROECOTOX is a Coordinating Action funded by the European Commission’s FP7 Environment Programme. The initiative is a European Network established to promote the integration of European activities on the replacement and reduction of animal experiments in ecotoxicology. EUROECOTOX aims to foster the exchange of knowledge, data, ideas and available European expertise to accelerate the research and development, (pre)validation and regulatory acceptance of alternative ecotoxicity tests and methods. The network is open to European R&D groups from universities, public research institutions, animal welfare groups and other non-profit organizations, specialized ecotoxicological contract laboratories, industry and other stakeholders developing alternative test methods.

Planned activities of EUROECOTOX include: Mapping of European research capacities on
alternative ecotoxicological test methods, identification of the rate-limiting steps to reduce or replace the use of animals, identification and promotion of new alternative methods, recommendations for future research, promotion of the dialogue with validation agencies and other coordination and networking activities.

Scientific Coordinator: Dr. Joaquín Guinea (ZF BioLabs S.L., Spain)

More information: http://www.euroecotox.eu/

OSIRIS: Optimised Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information

The project was already introduced in the first volume of this Annual Report. It was an Integrated Project funded under the European Commissions 6th Framework Programme and terminated in September 2011. The goal of the project OSIRIS was to develop integrated testing strategies (ITS) fit for REACH that enable a significant increase of the use of non-testing information for regulatory decision-making, and thus minimise the need for animal testing. For the first time, an ITS has been equipped with a decision theory framework including alternative methods such as: chemical and biological read-across, in vitro results, in vivo information on analogues, qualitative and quantitative structure-activity relationships, thresholds of toxicological concern and exposure-based waiving. The research of OSIRIS focussed on the following ITS:

- Skin Sensitisation
- Repeated Dose Toxicity
- Mutagenicity & Carcinogenicity
- Bioconcentration Factor
- Aquatic Toxicity

Scientific Coordinator: Gerrit Schüürmann (Helmholtz Centre for Environmental Research – UFZ, Germany)

More information: http://www.osiris.ufz.de/

The following list comprises titles and contact information of other related, currently running initiatives that were already introduced in the first volume of this Annual Report:

SC4SM: Stem Cells for Safer Medicines

More Information: http://www.sc4sm.org/
AXLR8: Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development

More information: http://axlr8.eu

ChemScreen: Chemical substance in vitro / in silico screening system to predict human- and ecotoxicological effects

More information: http://chemscreen.eu/

Predict-IV: Profiling the toxicity of new drugs: a non animal-based approach integrating toxicodynamics and biokinetics

More information: http://www.predict-iv.toxi.uni-wuerzburg.de/

ESNATS: Embryonic stem cell-based novel alternative testing strategies

More information: http://www.esnats.eu

Virtual Liver Network

More information: http://www.virtual-liver.de

Important institutions that are active in SEURAT-1 related fields are given in the following list (summary reports about them are also included in the first volume of this Annual Report):

ECVAM: European Centre for the Validation of Alternative Methods


CAAT-Europe: The Center Alternatives to Animal Testing - Europe

More information: http://cms.uni-konstanz.de/leist/caat-europe/

OECD Chemicals Testing - Guidelines

5.2.2 **International Activities**

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**USA**

**Integrated Microphysiological Systems for Drug Efficacy and Toxicity Testing in Human Health and Disease**

The National Institute of Health (NIH) published a call for proposal in November 2011. Applications for projects that will develop accurate cellular and organ microsystems representative of human physiology for the evaluation of drug efficacy and toxicity were invited. By definition, these cellular and organ microsystems will have a multicellular architecture representing the characteristics and functions of the tissue of origin and will demonstrate a reproducible and viable operation under physiological conditions over a long culture period. It is anticipated that these bio-engineered human tissue models could lead to the development and commercialization of microsystems that will enable rapid and high fidelity evaluation of safety and efficacy for candidate therapeutics.


**DrugMatrix: A Toxicogenomics and tissue library hosted by the National Toxicology Program**

DrugMatrix is the scientific communities’ largest molecular toxicology reference database and informatics system. It is a current project of the National Institute of Environmental Health Sciences – National Institutes of Health (NIEHS). DrugMatrix contains a graphic user interface for rapid scoring of genomic signatures of toxicity. DrugMatrix is populated with the comprehensive results of thousands of highly controlled and standardised toxicological experiments in which rats or primary rat hepatocytes were systematically treated with therapeutic, industrial, and environmental chemicals at both non-toxic and toxic doses and multiple exposure durations. The heart of the DrugMatrix database is large-scale gene expression data generated by extracting RNA from the toxicologically relevant organs and tissues and applying these RNAs 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 characterize mechanisms of pharmacological/toxicological action and identify potential human toxicities.


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 U.S. 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 organizations since its establishment in 1997. ICCVAM has also identified critical research, development, and validation efforts needed to further advance numerous other alternative methods.

In 2008 NICEATM and ICCVAM published a five-year plan for the years of 2008 through 2012. The current plan addresses (1) identification of areas of high priority for new and revised non-animal and alternative assays to reduce, refine (enhance animal well-being and lessen or avoid pain and distress), and replace the use of animals in testing, and (2) research, development, translation, and validation of new and revised non-animal and other alternative assays for integration into Federal agency testing programs. NICEATM and the National Institute of Environmental Health Sciences (NIEHS) and the invited public can provide comments that can be considered by the ICCVAM and agencies’ program offices in updating this five-year plan. A request for comments was published in the Federal Register on 21 November, 2011 (76 FR 71977). Comments were requested by 15 January, 2012.

More information: http://iccvam.niehs.nih.gov/
Other related initiatives that were already described in the first volume of the Annual Report are:

**Tox21: Toxicity 21**
More information: http://www.epa.gov/ncct/Tox21/

**ToxCast™: Screening Chemicals to Predict Toxicity Faster and Better**
More information: http://www.epa.gov/ncct/toxcast/

**ToxRefDB: Toxicity Reference Database**
More information: http://www.epa.gov/ncct/toxrefdb/

**v-Liver™: The Virtual Liver Project**
More information: http://www.epa.gov/ncct/virtual_liver/

**Other components of the EPA’s Computational Toxicology Research Program**
More information: http://www.epa.gov/ncct/research_projects.html

**PSTC: Predictive Safety Testing Consortium (InnoMed)**
More information: http://www.c-path.org/pstc.cfm

**HESI: Health and Environmental Sciences Institute**
More information: http://www.hesiglobal.org/

**CAAT: Centre for Alternatives to Animal Testing**
More information: http://www.caat.jhsph.edu

JAPAN

**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 5-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 Affymetrix GeneChip and collected together with classical toxicological data. Experiments were also done with rat and human hepatocytes and more than 8 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 utilize this system effectively, the second stage of the Toxicogenomics Informatics Project was started in 2007.

TG-GATES is publicly available (http://toxico.nibio.go.jp/open-tggates/search.html).

Further summary reports about the related Japanese Initiatives were given in the first volume of this Annual Report:

JaCVAM: Japanese Center for the Validation of Alternative Methods
More information: http://jacvam.jp

Percellome Project
More information: http://www.nihs.go.jp/tox/TTG_Archive.htm

5.2.3 Meetings and Symposia

Focus on Alternative Testing

European Partnership for Alternative Approaches to Animal Testing

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a joint initiative from the European Commission, European trade associations and individual companies. The EPAA organises Annual Conferences and workshops, which will be announced through its webpage (see below). Most important for the SEURAT-1 Research Initiative were the following activities:

Another workshop focussing on Computational Chemistry entitled “Revolutionising Toxicology. Developing a Research Prospectus”, which was also held in Brussels on 3 – 4 April 2012.

The latter built on the conclusions of the previous “Harnessing the chemistry of life” workshop held in July 2010. Experts at the leading edges of computational chemistry, systems biology, toxicology and related disciplines worked on the development of a research prospectus designed to inform responses to future calls for research in predictive toxicology. The workshop confirmed and built on the central theme of liver mitochondrial toxicity and agreed on the key elements of a research prospectus to make predictive, quantitative computational models possible. The prospectus would detail what could and should be done in ways that would complement the existing ongoing projects at the international level, what it would take to achieve this, and how the research should be organised and tightly integrated. Such a programme of research would:

- Consider transport and interactions from molecular to cellular/organelle levels (liver mitochondria specifically)
- Be tightly integrated with the in vitro experimentation designed specifically to inform this modelling activity
- Couple directly to systems modelling from cellular to organ level
- Take account of mechanistic understandings of toxic responses in the liver
- Build on the existing appropriate infrastructure for computation data basing and sharing.

The workshop underlined the importance of liver mitochondrial toxicology in the development of quantitative, predictive, toxicological models. The prospectus will be published by end of June 2012, the full workshop report is published on the EPAA homepage (http://ec.europa.eu/enterprise/epaa/2_activities/2_3_comm_and_dissem/comp-chem-flash-report.pdf)

More information: http://www.epaa.eu.com

Ecopa

Similarly, the European Consensus-Platform for Alternatives (ecopa) has been established to stimulate research into alternatives to animal experiments 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/
IVTIP: *In Vitro* Testing Industrial Platform

Date: 19 April 2012

Location: Bilbao, Spain

The *In Vitro* Testing Industrial Platform was established in December 1993 with the aim (i) to advise European bodies (European Commission, European Parliament) on industrial requirements for current and future research projects in *in vitro* testing; (ii) to optimise the industrial value of EU funded research projects; (iii) to encourage the further development, validation and regulatory acceptance of tests based on this research. IVTIP members represent companies in the following sectors: chemicals, cosmetics, consumer products and pharmaceuticals. IVTIP organises two plenary meetings per year, where its members meet with invited regulators, SMEs and academics active in the field of *in vitro* testing. During the meetings state-of-the-art presentations are given and position papers are drafted.

The meeting of April 19 is an open meeting on the Safety Assessment of Nanomaterials, meant for both IVTIP members and non-IVTIP members.

More information: http://www.ivtip.org/

Joint Convention on Scientific Roadmap for the Future of animal-free Systemic Toxicity Testing

Organisers: CAAT-Europe, CAAT-US, CEFIC, Cosmetics Europe, DZF, ECOPA, ESTIV, EUSAAT, IIVS, IVTIP, HSI, ToxCast

Date: 20 – 21 March 2012

Location: Brussels, Belgium

The desire to transition to animal-free systemic toxicity testing is fuelled by testing needs such as the European REACH regulation and a possible US TSCA reauthorization, as well as the testing bans for cosmetic ingredients in Europe. Other areas and novel products could similarly benefit from human predictive approaches. Recently, gaps in the available science had been identified. In order to promote the development of a roadmap to close these gaps, an expert workshop was held and presented. The proposed roadmap was discussed during the joint convention in a multi-stakeholder forum.

SETAC: 6th World Congress

Date: 20 – 24 May 2012

Location: Berlin, Germany
The society of Environmental Toxicology and Chemistry (SETAC) is a non profit, global professional society established in 1979 to provide a forum for individuals and institutions engaged in education, research and development, ecological risk assessment and life-cycle assessment, chemical manufacture and distribution, management and regulation of natural resources, and the study, analysis, and solution of environmental problems. Besides other topics on environmentally related issues, the 6th World Congress, entitled “Securing a sustainable future: Integrating science, policy and people”, also comprised a special session on “Animal Alternatives and Testing Strategies”, as well as a regular session on

- Animal Alternatives: Methods, Endpoints and Testing Strategies
- A Systems Biology Approach to Predictive Ecotoxicology
- Quantitative Structure Activity Relationship (QSARs) and similar Models for Predicting the Toxicity of Chemicals, Mixtures and Combined Stress
- Approaches for Comparative Hazard and Risk Assessment of Chemicals
- Standard versus Non-standard Methods for Hazard and Risk Assessment

More information: http://www.berlin.setac.eu/

INVITROM: Advances in In-Vitro Cell and Tissue Culture

Date: 22 – 23 May 2012
Location: Utrecht, The Netherlands

INVITROM is the “Dutch-Belgian Society for In Vitro Methods”. Their mission is the promotion of the development, application and acceptance of the in vitro models in the biomedical research. The ambition of INVITROM is to accelerate the development of models, paradigms and strategies through information exchange. Moreover, INVITROM wants to promote the development, acceptance and implementation of these methods amongst others, by stimulating the collaboration between the research institutes and industry and by informing the regulatory bodies. In order to achieve these goals, INVITROM uses several communication tools including workshops and symposia.

The 2012 INVITROM meeting was combined with the 4th Annual Quasi-Vivo® User Group Meeting and provided a forum for Europe’s leading researchers to present their in vitro cell culture advances and exchange ideas. It was a ‘Gordon Conference Style’ event at which everyone who attended was encouraged to contribute either by presenting papers, preparing a poster or running a discussion session. Prizes were presented to the best student posters.

Main themes for discussions were:

- Pharmaceutical Drug Discovery and Development
AXLR8: Workshop 2012

Date: 10 – 13 June 2012
Location: Berlin

“Roadmap to Next Generation Safety Testing under Horizon 2020” was the title of the 2012 AXLR8 workshop. AXLR8 is a coordination action funded by the European Commission Directorate General for Research and Innovation under the Health Theme of the 7th European RTD Framework Programme. The project intends to accelerate the transition to a toxicity pathway-based paradigm for chemical safety assessment through networking activities, information exchange, strategic planning and collaboration among a variety of scientific disciplines and stakeholder groups. In this context, the yearly-organised workshop plays a central role for fostering the network activities.

Members of COACH from the SEURAT-1 Research Initiative participated in the workshop and presented the first results that are also published in this Annual Report (see chapter 4). The workshop is a very important platform for establishing future cooperation, not only with other European initiatives, but also with the related activities in the USA and Japan.

More information: http://axlr8.eu/

EUROECTOTOX: 1st European Conference on the Replacement, Reduction and Refinement of Animal Experiments in Ecotoxicology

Date: 28 – 29 June 2012
Location: Dübendorf, Switzerland
The conference aims to provide a platform for young scientists and experts from academia, industry and regulation in the field of 3Rs in environmental risk assessment with a focus on the current state and future directions of the development, implementation and application of the 3Rs, from bench to regulatory acceptance.

Main themes for discussions were:

➤ Experimental approaches (model systems; mechanisms of toxicity; adverse outcome pathways; test set-ups and standardization; concepts of in vivo-in vitro relationships)

➤ Computational approaches (physiologically based quantitative computational models; computational systems biology; quantitative structure activity; structural alerts)

➤ Integrated testing strategies (high throughput screening; visualisation and quantitative analysis; linking chemical exposures and effects; in silico methods)

More information: http://www.euroecotox.eu/

Mondial Research Group meeting on Reduced Animal Testing

Date: 26 – 27 July 2012

Location: Zurich, Switzerland

Although most of the experiments performed on animals are regarded as important for the furtherance of human and veterinary science, there is a strong movement from within the scientific community to develop methods that do not rely on animals. However, it still may take a long time before all animal experiments can be replaced. The meeting focuses, therefore, on options to reduce both the number and suffering of experimental animals.

Main themes for discussions were:

➤ An in-depth study of the 3R’s

➤ Relative and absolute replacement models

➤ Difficulties of extrapolating results to the human situation

➤ In vitro methods: Replacement or addition to animal testing

➤ Computer modelling, biochemical techniques and in vitro methods

➤ The refinement and reduction of suffering of experimental animals before, during and after an experiment

More information: www.mondialresearchgroup.com/
OTHERS IN THE FIELD

Metabolomics in Toxicology and Preclinical Research: State-of-the art and potential applications

Organisers: BASF SE and Centre of Alternatives to Animal Testing - Europe (CAAT-Europe)
Date: 13 February 2012
Location: Berlin, Germany

This Symposium brought together scientists from academia, industry and regulatory bodies to present the current status of the metabolomics technology and its applicability in toxicology, particularly for safety assessment of compounds. Metabolomics, as compared to other ‘-omics’ approaches, can be considered as being the closest of the ‘-omics sciences’ to classical toxicology, while providing information on a high level of integration.

Main themes for discussion were:

- Improvement of our understanding about the toxicological profile of a given compound; i.e. identifying its toxicological mode of action(s)
- Identification of biomarkers that can potentially be used to identify pathophysiological conditions or, in cases of drugs used for treatment, monitor efficacy of treatment
- Identification of biochemical pathway changes following exposure.

More information: http://www.ivtip.org/

51st Annual Meeting of the Society of Toxicology (SOT)

Date: 11 – 15 March 2012
Location: San Francisco, USA

The SOT Annual Meeting is the most comprehensive forum to highlight premier scientific presentations that span the discipline of toxicology. From the essential knowledge to the latest advances, the scientific sessions, including platform sessions, poster presentations, and plenary talks, provide access to the important information of the field.

Main themes for discussion were:

- Aberrant Gene Expression in Toxicity and Disease—Epigenetics and MicroRNAs
- Characterizing Toxic Modes of Action and Pathways to Toxicity
- Identification of biochemical pathway changes following exposure
Clinical Toxicology from Bedside to the Bench and Back
Influence of Global Climate Change on Environmental Health Issues
Regulatory Science: Bridging the Gap between Discovery and Product Availability

More information: http://www.toxicology.org/ai/meet/am2012/

11th Annual World Pharma Congress (WPC)
Organisers: Cambridge Healthtech Institute
Date: 05 – 07 June 2012
Location: Philadelphia, USA

The conference focused on pre-clinical efforts targeted towards early discovery, screening and safety assessments. It brought together a mix of scientists and clinicians from academia and industry to facilitate active brainstorming and networking on challenging issues in the field. The conference also included contributions from leading technology and service providers on the latest tools and services available in the marketplace. The Congress motto 2012 was: “Promising Assays and Technologies for Better Pre-Clinical Predictions.”

Main themes for discussions were:

- Predicting Drug-Induced Cardiotoxicity
- Targeting Alzheimer’s Disease
- Predictive Pre-Clinical Models in Oncology
- Tackling Drug-Induced Idiosyncratic Hepatotoxicity
- Molecular Imaging in Drug Discovery and Development
- Targeting Pain with Novel Therapeutics

More information: http://www.worldpharmacongress.com/

48th Congress of the European Societies of Toxicology
Date: 17 – 20 June 2012
Location: Stockholm, Sweden

The Federation of European Toxicologists & European Societies of Toxicology (EUROTOX), with about 7000 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 to encourage future work in toxicology (scientifically as well as educationally).

Main themes for discussions were:

- Chemical exposure-related inflammation and cancer
- Innovative testing strategies to identify chemical respiratory sensitizers: present and future
- Neurotoxicology of metals: mechanisms and clinical effects
- Mechanisms of cell death and survival
- Mixture toxicity: Current approaches and future strategies
- From mechanisms of toxicity to biomarkers: addressing current and future needs in drug safety assessment
- Role of immunosurveillance in chemical carcinogenesis
- Large populations at risk? News on adverse health effects of low dose exposure to toxic metals
- From Nanotoxicological Research to Safe Management of nanomaterials
- Unravelling the natural functions of the aryl hydrocarbon receptor and its proposed endogenous ligands
- Read-across in risk assessment; problems or possibilities?
- Pollution from drug manufacturing – assessing and managing risks in different regions of the world
- New mechanistically based models for evaluation of drug induced liver injury: the IMI Predictive DILI project
- Clinical toxicology: Are new insights into epidemiology and mechanisms of toxicity changing our approach to important poisonings?
- Pharmaceuticals in the environment: occurrence, effects on wildlife, and how to reduce the levels
- Toxicological significance of pharmacogenomics in cancer treatment
- Dose-response relationship and receptor-mediated toxicology
- Stem cells in drug discovery and development

Evidence-based Toxicology Collaboration Europe

Date: 17 June 2012
Location: Stockholm, Sweden

Following the US effort of creating an Evidence-based Toxicology Collaboration (EBTC) in 2011, a European counterpart to adapt Evidence-based Medicine (EBM) principles to Toxicology just started. Evidence-based Toxicology seeks to systematically implement transparency, objectivity, and consistency in toxicology. The EBM toolbox (e.g., systematic reviews and test assessment methodology) is available for translation to toxicology, as are the approaches of the Cochrane Collaboration, which applies and fosters systematic reviews. Interested scientists were invited to the official kick-off of the European branch of the EBTC and to become part of the collaboration. The kick-off meeting of EBTC Europe took place in conjunction with the Eurotox Congress 2012.

Euroscience Open Forum 2012

Date: 11 – 15 July 2012
Location: Dublin, Ireland

The Euroscience Open Forum (ESOF) is Europe’s largest general science meeting and is held in a leading European city every two years. It is an interdisciplinary, pan-European meeting, held under the auspices of Euroscience, which aims to (i) showcase the latest advances in science and technology; (ii) promote a dialogue on the role of science and technology in society and public policy; (iii) stimulate and provoke public interest, excitement and debate about science and technology.

The science programme will comprise interactive seminars, workshops, panel discussions and debates on fundamental questions encapsulating the essence of the following seven overarching themes for the meeting:

- The Future of Medicine & Health
- Reshaping the Frontiers of Knowledge
- Energy, Environment & Climate
- Engagement & Education
- Communicating Science
- Science & Culture
- Research Policy

ESTIV2012: International Conference of the European Society of Toxicology *In Vitro*

Date: 16 – 19 October 2012  
Location: Lisbon, Portugal

The European Society of Toxicology *In Vitro* is the leading organisation in Europe that strengthens the scientific network of the *in vitro* toxicologists and promotes *in vitro* toxicology, both scientifically and educationally, in all countries of Europe. As in the previous ESTIV events, this conference will bring together researchers and students from academia and industry, involved in the development and use of *in vitro* methods in toxicology.

ESTIV 2012 will cover a broad range of topics addressing systemic toxicity, local toxicity and developmental toxicity, with the emphasis on physiologically relevant markers, marker profiles, molecular mechanisms and pathways. For the first time and in addition to the cutting-edge topics that will be covered in the conference, a practical workshop will be organized on the 20th October. The purpose of this workshop is to gain a hands-on experience with computerised *in vitro – in vivo* extrapolation strategies.

More information: http://www.estiv.org/

52nd Annual Meeting of the Society of Toxicology (SOT)

Date: 10 – 14 March 2013  
Location: San Antonio, USA

The SOT Annual Meeting is the most comprehensive forum to highlight premier scientific presentations that span the discipline of toxicology. From the essential knowledge to the latest advances, the scientific sessions, including platform sessions, poster presentations, and plenary talks, provide access to the important information of the field.

Main themes for discussion will be:

- Application of Systems Biology to Toxicology
- Biomarker for Exposure Assessment, Safety Evaluation, and Translational Medicine
- Effects of Nanomaterials on Biological Systems
- Molecular Basis of Genetic Variability and Susceptibility to Toxicants
- Influence of Global Climate Change on Environmental Health Issues
- Regulatory Science: Advancing New Approaches for Hazard Identification and Risk Assessment

5.3 Developing in vitro Tools Sufficient by themselves for 21st Century Risk Assessment

Melvin E. Andersen, Rebecca Clewell and Sudin Bhattacharya

Abstract

The field of toxicity testing for non-pharmaceutical chemicals is in flux with multiple initiatives in North America and the EU to move away from animal testing to mode-of-action based in vitro assays. In this arena, there are still obstacles to overcome, such as developing appropriate cellular assays, creating pathway-based dose-response and in vitro-in vivo extrapolation (IVIVE) tools, and providing assurances that new approaches are adequately protective of human and ecological health. Another major challenge for individual scientists and regulatory agencies is developing a cultural willingness to shed old biases developed around animal tests and become more comfortable with mode-of-action based assays in human cells. At present, most initiatives focus on developing in vitro alternatives and assessing how well they reproduce past results by predicting organism level toxicity with intact animals. However, the path forward actually requires looking beyond benchmarking against high dose animal studies. We need to develop targeted cellular assays, new cell biology-based extrapolation models for assessing regions of safety for chemical exposures in human populations, and mode-of-action-based approaches to compare data generated from in vitro methods with results from animal studies as the tie to past practices. Our home organization, The Hamner Institutes for Health Sciences, is working to develop several pathway-targeted case studies. The projects include p53-mdm2-mediated DNA-damage, estrogen receptor, PPARa receptor, and Nrf2-Keap1 oxidative stress response pathways. These case studies will produce a mechanistic understanding of the molecular circuitry for specific toxicity pathways, develop computational systems biology pathway (CSBP) models for each case study pathway, and apply the knowledge of pathway circuitry and dynamics to support safety assessments for groups of compounds affecting the case study pathways. We describe the rationale for this case study-based approach, and provide a short description of the status of Hamner activities with the selected case studies.

5.3.1 Background

The National Research Council (NRC) report from the US National Academy of Sciences, Toxicity Testing in the 21st Century: A Vision and A Strategy, proposed a shift from toxicity

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testing using animal studies to evaluation of perturbation of toxicity pathways in mode-of-action-based *in vitro* assays using human cells or human cell lines (NRC, 2007; Krewski *et al.*, 2010). There were already several other initiatives to develop alternatives to animal testing and tiered approaches to reserve animal testing for those chemicals more likely to have specific forms of toxicity. The NRC report was essentially different from these other approaches in stressing that these new *in vitro* methods were the preferred approach for toxicity testing of environmental compounds.

In the USA, the EPA’s ToxCast program, in collaboration with the Tox21 initiative and other research partners has developed approaches to screen chemicals through a diverse suite of assays using quantitative high throughput screening (q-HTS). Phase I of the ToxCast program included assays for over 300 compounds. The express goal of the program was to develop bioactivity signatures that would assist in prioritizing compound for further testing. Phase II has 1,000 chemicals from a broad range of sources including industrial and consumer products, food additives and drugs and will evaluate the predictivity of toxicity signatures developed in Phase I. Many papers describing the ToxCast research are now available (Judson *et al.*, 2010; Martin *et al.*, 2010; Kleinstreuer *et al.*, 2011; Martin *et al.*, 2011; Sipes *et al.*, 2011). Even though the predictive potential of the assays for in-life toxicity in Phase I appears low (Thomas *et al.*, 2012), there are proposals for using ToxCast results to estimate “Toxicity-Related Biological Pathway Altering Doses for High-Throughput Chemical Risk Assessment” (Judson *et al.*, 2011). Many of the EU-based initiatives focus more on animal alternatives, especially in light of the restrictions on using animals to test safety of cosmetics.

What is clear is that the future path for toxicity testing will differ significantly from the past efforts. Newer *in vitro* methods would allow broad evaluation of dose-response including concentrations equivalent to those arising from ambient human exposures. The read-out of the assays would include measures of adverse responses *in vitro* and the dose response for the pathway, to support pathway-based dose-response modeling (Boekelheide & Andersen, 2010). The goal of these alternative approaches will not be to predict high dose responses in test animals. Instead, they will assess regions of safety and modes-of-action. From knowledge of modes of action, it would be possible to say something like, “Due to the pathways targeted by the chemical, high level exposures to the test compounds may affect some specific tissues or organ systems. However, no tests have shown such responses in animals or people”. It will be challenging to convey this type of information to the public. Nonetheless, the “Pathway Altering Dose” concept and the proposed use of ToxCast profiles to develop so-called chemical-specific Provisional Peer Reviewed Toxicity Values (PPRTVs) for chemicals associated with natural gas recovery through fracking show some intention by US EPA to use *in vitro* screening in a more risk assessment oriented manner.

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5.3.2 Outlining a Process

How will toxicologists and risk assessors use results from mode-of-action-based toxicity pathway assays to estimate regions of safety with various chemicals? The NRC report outlined a deliberate process to develop a variety of assays and modeling tools over a 10 to 20 year period to prepare for a day when the risk assessment machinery in the US would change radically from current practice to a toxicity pathway foundation. In retrospect, this suggestion appears naïve. The process needs to move along piecemeal, developing examples of the use of toxicity pathway approaches on a more limited scale, in order to provide illustrative examples of a new process in action and to enhance confidence in making changes. The use of case studies with a group of pathways provides an opportunity to develop tools, apply them for specific modes-of-action, and show their use in risk/safety estimations for chemicals affecting these pathways (Andersen et al., 2011). In this manner, the assays come on line incrementally along with key extrapolation tools. Current Hamner research includes six toxicity pathway case studies – three receptor-mediated pathways (for the aryl hydrocarbon (AhR), the peroxisome proliferator-activated receptor-alpha (PPARα), and the estrogen receptor (ER)) and three reactivity-based pathways (DNA-damage, oxidative stress and mitochondrial toxicity).

![Figure 5.1 Data and Modeling in creating ‘validated’ mode-of-action-based toxicity pathway assays. Starting with an assay selected to probe a particular mode-of-action, dose and time-course studies with positive control chemicals for the pathway (3 ellipses on the left) coupled with knowledge of pathway circuitry produce a computational pathway model (center). IVIVE methods convert the active in vitro concentration to exposures levels that would produce these responses in a laboratory or real world exposure. The output of this analysis permits comparison with animal studies that examine similar mode-of-action markers to determine if such responses occur at similar concentrations in vitro and in vivo.](image-url)
The goal of all these case studies is to create a workflow where carefully designed and validated assays measure perturbations of specific pathways indicative of particular modes of action. The first stage of assay development is selection of the cell system for use; the second consists of collection of a data stream to unravel the circuitry and dynamic properties of the pathway (Figure 5.1). The aggregation and bioinformatic analysis of these data streams creates a quantitative picture of pathway circuitry and pathway dynamics across a range of perturbations. There are several good descriptions of circuitry and dynamics as they relate to biological systems (Strogatz, 2000; Alon, 2007; Tyson & Novak, 2010). These pathway models support dose response extrapolations. In this approach of data collection and analysis, the process of validation changes from asking whether the assays recapitulate high-dose animal responses into a two-pronged procedure. First, we ask, does the assay capture the expected dose-response for positive control compounds affecting these pathways? Second, how does the dose-response developed from these validated assays compare with that observed in mode-of-action oriented in vivo studies with the positive control compounds? These steps, shown to the right in the figure, link together through our emerging understanding of biological control theory and systems dynamics arising from advances in computational cell biology to inform the process of setting exposure standards for human populations.

Over the past 5 years, Hamner staff in the Center for Dose Response modeling have developed modeling tools to assess aspects of contemporary computational cell biology that are likely key to understanding cellular control mechanisms and dose response. To further advance the acceptance of these tools, we developed a course in “Computational Systems Biology and Dose Response Modeling”. One recent offering was in early May 2012 through collaboration between the Hamner and the Institute for Health and Consumer Protection, European Commission, DG Joint Research Centre, Ispra, Italy. As much as dissemination of modeling tools, these courses have provided a greater appreciation of the control processes and circuits by which cells respond to normal signaling cues and to perturbations by various environmental stressors. Aspects of nonlinear dynamics and control theory, from considerations of homeostasis with negative feedback to bistability arising from ultrasensitivity in hormonal signaling processes, provide a theoretical background for considering perturbations of cell signaling pathways by chemicals. The Hamner has also developed teaching material for IVIVE approaches in a course called “Physiologically Based Pharmacokinetic (PBPK) modeling and in vitro-in vivo Extrapolation”. All the materials – lectures, laboratory exercises and computational models used in the exercises – are on the Hamner web site for open usage.

5.3.3 Mode-of-Action based Assays

Considerable effort and research will be necessary to validate an assay. However, this level of detail supports the initial assay development and validation. The routine use of assays, as part of a battery, is likely to be much simpler (Figure 5.2). Here information from a variety of

4 - http://www.thehamner.org/pbk-course-2010
test modalities, such as quantitative high-throughput screening (q-HTS), high content imaging assays (HCA) and quantitative structure activity relationship (QSAR) modeling, indicate expected pathway targets, identifying one or a limited number of mode-of-action based pathway assays for follow-up. The subsequent testing for these pathways probes a limited number of read-outs defined after completing the validation process (from Figure 5.1). The read-outs, depending on the pathway, may be a truncated set of genes, specific phosphoprotein panels, cellular response phenotypes, or various HCA providing specific cell response measures. These individual pathway tests may lend themselves to development as commercial test kits in the near future. The integrated assay results together with CSBP models would provide the ‘acceptable’ in vitro concentration. Then, together with policy considerations, such as sensitive populations and response variability in a diverse population, adjustments to the in vitro adverse concentration give an acceptable human plasma concentration. The last step in this process, estimating the in vivo human exposure expected to produce the in vitro concentration, would rely on quantitative in vitro-in vivo extrapolation - QIVIVE (Shiran et al., 2006; Gibson & Rostami-Hodjegan, 2007) - or reverse dosimetry (Clewell et al., 2008). A higher throughput procedure, referred to as reverse toxicokinetics, has examined the expected kinetics of a large number of Phase I compounds (Rotroff et al., 2010; Wetmore et al., 2012).

![Diagram](image)

**Figure 5.2** Work-flow for toxicity testing leading to use of specific toxicity pathway assays. Validated assays support testing in a tiered approach. A suite of screening and higher throughput studies (beige ellipses) provides data that identifies the need for conducting specific assays. The results of these various studies couple through the CSBP and QIVIVE models to predict regions of safety for the chemical.

### 5.3.4 Progress

The following section provides an overview of current Hamner projects, including the development of research tools for studying toxicity pathways and application of the tools with specific case studies.
Genomic Tools

One of our first genomic dose response studies *in vivo* evaluated the dose response for nasal epithelial gene expression with formaldehyde across multiple exposure concentrations (0, 0.7, 2, 6, 10, and 15ppm) and multiple durations of exposures from 1-day up to 3 months (*Andersen et al., 2008; Andersen et al., 2010*). Analysis of the shorter-term formaldehyde exposures produced a method for creating benchmark dose (BMD) values for alterations in expression of genes within gene ontology (GO) categories (*Thomas et al., 2007*). The bioinformatic tools for establishing BMDs for genes within GO-categories (*Yang et al., 2007*) are available at http://sourceforge.net/projects/bmdexpress/. These genomic tools examine responses for toxicity pathways *in vitro* and in targeted short-term *in vivo* exposures (*Thomas et al., 2011; Black et al., 2012*). As we move toward pathway validation, the genomic tools need to expand from benchmark evaluations to support mechanistic evaluation of pathway function and network inference (*Shen et al., 2011*).

PPARα Pathway Studies:

Activation of the peroxisome proliferator-activated receptor alpha (PPARα) nuclear receptor in liver parenchymal cells results in a series of coordinated events leading to downstream alterations in gene expression with alterations in lipid and fatty acid metabolism. For the past three years, The Hamner has received funding to map and model the PPARα signaling pathway in primary hepatocytes from human and rat and has participated in the OECD toxicogenomics and molecular screening project. This pathway represents a prototype of a nuclear receptor mediated toxicity pathway with important species differences. We have used a combination of microarray-based gene expression data, regulatory interactions inferred from protein-DNA transcription factor arrays and published CHIP-on-chip (chromatin immunoprecipitation followed by microarray hybridization) results (*van der Meer et al., 2010*) to develop a picture of PPARα-mediated transcriptional regulation after treatment with the PPARα specific ligand GW7647. This agonist altered expression of about 200 genes in human primary hepatocytes and nearly 500 in rat primary hepatocytes. Only a limited number of genes were direct genomic targets of PPARα. We then inferred the transcription factors (TFs) involved in gene regulation, leading to a clearer picture of the hierarchical organization of the PPARα response network and the concentration- and time-dependent structure of the network.

The inferred response network will serve as the basis for quantitative computational models of the PPARα pathway. The sequential regulation of genes lacking PPARα binding may occur through phosphorylation cascades initiated by GW7647 binding to PPARα (*Diradourian et al., 2005; Burns & Van den Heuvel, 2007*). We have also added kinases into the provisional network through use of publicly available databases5 and linked PPARα through these kinases with the TF network. Our working hypothesis with PPAR signaling is that the receptor after activation by GW7647 activates a bistable switch (*Figure 5.3*) with the requisite ultrasensitivity

5 - www.kinasource.co.uk/Database/welcomePage.php
of the switch arising from mitogen-activated protein kinase (MAPK) cascades. The switch, affecting activation of gene batteries by PPARα, would be reversible with cessation of agonist treatment. Bistable switches give rise to steep-dose response in individual cells and responses need to be measured both in populations of cells and in individual cells to assess the behavior. Several studies with liver nuclear receptor activation already support all-or-none responses (Bars et al., 1989; Tritscher et al., 1992; Andersen & Barton, 1999).

Figure 5.3 Receptor-mediated regulation of hepatic biology may rely on switch-like behaviors. Induction of cytochrome proteins in liver cells can occur in an all-or-none fashion. As the dose of beta-naphthoflavone (BNF) increased (right panel), more hepatocytes became induced (Bars et al., 1989). In terms of pathway dynamics, a bistable switch (left panel) can account for this behavior where kinase cascades support the bistability. Our studies will determine whether bistability underlies the induction of proteins in liver by PPARα agonists and the identity of the kinases creating the bistability.

p53 –DNA Damage Pathway Research

In collaboration with scientists from Unilever-UK, Dr. Rebecca Clewell and her laboratory staff study the p53-mdm2 DNA damage response networks in human cells to determine the dose response behavior for activation of the p53 pathway after chemically induced DNA damage and the underlying response circuitry for this pathway. The p53 research has two overarching goals: (1) to map the key determinants of cellular fate following DNA damage induced by chemicals with different mechanisms of action (indirect vs. direct DNA-damage) and (2) to identify dose-dependent thresholds associated with cellular adaptation and toxicity.
(and mutation) after chemical-induced DNA damage. This project has collected a dense data stream for inferring the structure and dynamics of the DNA-damage toxicity pathway. Currently, multi-dose, multi-time transcriptomics, high-throughput flow cytometry (HTFC) and high-content imaging (HCI) technologies examine the gene and protein response of the p53 DNA-damage networks, as well as cell cycle progression and cell death for three chemicals: methylmethane sulfonate (MMS), etoposide (ETP), and quercetin (QUE) in two p53 competent human cell lines (HT1080 and AHH-1). Initial studies examined the time and dose-dependence of (1) the DNA damage marker p-H2AX, (2) whole genome mRNA expression, (3) targeted protein expression (p53, p-p53 (phosphorylated at ser15), MDM2, Bcl2), and (4) various phosphorylated kinases. Subsequent studies focused on cellular fate after differential DNA damage, including measures of fixed DNA-damage (micronuclei), cell cycle arrest, and apoptosis. A second stage of research will confirm the network structure through targeted knock down of key nodes – such as kinases and TFs – or overexpression of pathway proteins and provide quantitative results to assist in developing a threshold model for homeostasis as related to DNA-damage and mutation.

Current computational pathway models for p53 and DNA damage involve double negative feedback, oscillations and non-linear signal transduction (Lahav et al., 2004; Geva-Zatorsky et al., 2006; Batchelor et al., 2008; Lahav, 2008) with linkages between pathway activation and cell cycle arrest and apoptosis (Toettcher et al., 2010). We have implemented these DNA-damage models in a convenient software package, Berkeley-Madonna™. As with PPARα, we have a working hypothesis regarding the p53 pathway function (Figure 5.4). Endogenous levels of p53 are sufficient to handle small increases in DNA-damage (we measure functional outcome by micronuclei formation). With these small increases, regulatory molecules involved in activation of p53 by kinases, such as ATM, enhance the rate-constant for repair through a post-translational, feed forward loop keeping the dose-response curve flat. With increasing DNA-damage (consistent with the sloped portion of the right panel), control shifts from primarily post-translational responses to enhanced transcriptional control. These coupled integral feedback and feed forward loops are likely at work with all canonical stress pathways (Simmons et al., 2009). Work on the high-osmolarity glycerol (HOG) pathway in yeast is the best example of unraveling the network topology for canonical stress pathways showing parallel control modules (Mettetal et al., 2008; Muzzey et al., 2009).
Figure 5.4 Homeostasis in stress-controlling pathways requires multiple feedback processes. Feedback loops with ultrasensitivity and high loop gain control transcriptional responses to stressors - left panel (Zhang & Andersen, 2007). Feed forward processes with post-translational regulation (in blue) along with integral control (shown along the (Y) to (T) path assist in creating so-called ‘perfect’ control where there is a region of unchanged slope compared to background with increasing stressor (in this case showing hypothetical curves for micronuclei versus a dose of etoposide. Our studies cover the broad dose range, from maintenance of no greater than background micronuclei rates along the flat part of the curve on the right through increases in micronuclei and altered gene transcription with the increasing slope phase of the curve. The controlled variable would be various types of DNA-damage.

5.3.5 Other Pathway Projects

In 2012, The Hamner started a case study related to ER-signaling – a multi-PI program on estrogen-signaling pathway activation in uterine cells. The Hamner project closely follows Figure 5.1. The full proposal is on the Hamner web-site and contains the rationale for developing a mode-of-action-based test assay and plans for its application in risk assessment with estrogenic compounds. Support for the ER project is from a group of sponsors and represents an endeavor to map and model the pathway in sufficient detail to derive in vitro tools that permit a human health risk assessments with estrogenic endocrine disruptors. That is, after completion of the validation work on the pathway assay, the results of assay with other

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test compounds will guide a safety assessment without the need to move to in-life toxicity studies. This project both develops an integrated in vitro model for uterine cell responses and compares results with in vivo assays of uterotropic responses in the rat. The Hamner is also a co-investigator on a project housed at Johns Hopkins University looking at estrogen signaling in MCF-7 cells – a cell-line derived from a human breast cancer. The two projects have very significant synergies in identifying the signaling pathways for E2 in human tissues. Finally, The Hamner is also working with Unilever to develop an Nrf2-Keap1 oxidative stress pathway project. Our principal investigators have both laboratory and modeling experience working with this pathway (Zhang et al., 2009; Pi et al., 2010; Zhang et al., 2010).

5.3.6 Summary

On a modest scale, The Hamner toxicity pathway programs try to cover many areas comparable to the projects within the SEURAT-1 Research Initiative - designing assays, developing modeling tools for extrapolation, improving training, developing case studies, engaging stakeholders, and working with regulators. A major difference remains the primary emphasis of the overall programs. In our case, we plan to create mode-of-action based approaches that will predict regions of safety. Our goal is not to accurately predict dose-response behaviors for apical responses in animals exposed to high doses. Nonetheless, the processes of goal setting and careful management of the interdisciplinary team are key components to insuring progress and accountability in any program. We also strongly believe that the case study approach is essential to accelerating the transition to new testing modalities. Case studies show how the new methods will work in practice and tell us if key technology gaps need filled to make the approach using mode-of-action-based toxicity pathways assays feasible. At least, to the authors of this short paper, it is clear that our early investment in training through our courses in computational systems biology and QIVIVE helped shape our research programs. This training, educational component guided generation of hypotheses for pathway structure and function and the lab work.

Acknowledgments

The ACC-LRI (Long Range Research Initiative of the American Chemistry Council), Dow Chemical, Unilever, the ExxonMobil Foundation and Dow Corning have supported method development and data generation needed in pursuing case study approaches to bringing the TT21C vision to life more quickly.

7. Clarifying remark from the Editors: Even though anchoring of the in vitro and in silico results from the SEURAT-1 Research Initiative to apical responses in animals will be necessary in the initial stage of the programme, SEURAT-1 is not aiming to accurately predict apical responses in animals at high doses on a longer run. Instead, from our perspective, we are aiming at the same goal as the authors of this contribution.
References


3Rs
Reduction, replacement, refinement - defined by Russel & Birch 1959

ADME
Absorption, Distribution, Metabolism, and Excretion. ADME describes the disposition of a pharmaceutical compound within an organism (see also TK, toxicokinetics).

ADMET
Absorption, Distribution, Metabolism, Excretion, and Toxicity of a compound.

Analogue and / or category approach
The terms category approach and analogue approach describe techniques for grouping chemicals. The term analogue approach is used when the grouping is based on a very limited number of chemicals, where trends in properties are not apparent. A chemical category is a group of chemicals whose physicochemical and human health and/or environmental toxicological properties and/or environmental fate properties are likely to be similar or follow a regular pattern as a result of structural similarity (or other similarity characteristic). In principle, there should be sufficient members in the chemical category, to enable the detection of trends across endpoints. As the number of chemicals being grouped into a category increases, the potential for developing hypotheses and making generalisations about the trends will also increase, and hence increase the robustness of the evaluation.

AOP
An Adverse Outcome Pathway (AOP) describes and formalises the documented, plausible, and testable processes by which a chemical induces molecular perturbations which may lead to a toxic effect. As such it links directly to the associated biological responses which describe how the molecular perturbations cause effects at the subcellular, cellular, tissue, organ, whole animal, and population levels of observation. The AOP can then be used to form chemical categories to allow for read across (if appropriate). The AOP can be supported by knowledge of how chemicals interact with biological systems (i.e., the molecular initiating events) and in vitro and in vivo knowledge of the biological responses.

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.

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.

**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.

**Chemical category**
see Analogue and / or category approach.

**ChIP**
Chromatin Immuno-Precipitation, antibody based enrichment 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

**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

**DEB**
Dynamic Energy Budget

**EB**
Embryoid body

**EC**
Endothelial cell
**EC_{50}**
Half maximal Effective Concentration

**ECG**
Electrocardiogram

**ECHA**
European Chemicals Agency

**ecopa**
European Consensus Platform for 3R Alternatives

**ECVAM**
European Centre for the Validation of Alternative Methods

**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/

**ER stress**
Endoplasmatic Reticulum stress

**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

**GCCP**
Good Cell Culture Practice

**Gesicles**
Methodology for producing proteins and transferring them to target cells, based upon the introduction in producing cells of the gene encoding the viral fusogenic protein VSVG. Vesicles ("Gesicles" where the G
stands for the G viral protein) formed and released by those producing cells are, then, both much more numerous and very prone to fusion with cell membranes. Engineering producing cells with constructs encoding proteins of interest leads to packing of well translated and processed proteins in gesicles, providing a way to produce and transfer proteins into target cells where normal function has been well demonstrated.

GFP
Green fluorescent protein

GLP
Good laboratory practice

GMP
Good manufacturing practice

Gold Compound
A well characterised compound for toxicity testing.

HBV
Hepatitis B virus

HCC
Hepatocellular carcinoma

HCV
Hepatitis C virus

Hep G2cells
A HCC derived human hepato-carcinoma cell line (ATCC No. HB-8065) from liver tissue of a 15 year old Caucasian American male with a well differentiated hepatocellular carcinoma.

HepaRG cell line
HepaRG is an immortalized cell line of the liver that can be differentiated into hepatocytes which retain many characteristics of primary human hepatocytes.

hES cell
Human embryonic stem cell

hiPS cell
Human induced pluripotent stem cell

HLC
Hepatocyte like cell

HOMO
Highest Occupied Molecular Orbital

HSC
Hepatic stellate cells
HSEC
Hepatic sinusoidal endothelial cells

HTS
High-Throughput-Screening

IC10
10% inhibitory concentration

INCI
International Nomenclature of Cosmetic Ingredients

*In silico methods for toxicity prediction*
The use of computer-based methods e.g. databases, (Q)SARs, read-across etc to retrieve or estimate toxicological effects of chemicals. These do not require the testing of a chemical (and hence can be termed non-testing information).

Intermediate precursors
Cells that are committed to a specific lineage but are not terminally fully differentiated and exhibit the capacity to self-renew without changes in phenotype for a number of passages when grown in culture with specific cocktails of cytokines (e.g. EGF/FGF2 for neural precursors). Intermediate precursors can be terminally differentiated into discrete populations of their lineage. For SCR&Tox purposes, intermediate precursor populations are currently available in the neural, mesodermal and keratinocyte lineages

Interoperability
The ability of two or more systems or components to exchange information and to correctly use the information that has been exchanged. More generally, it is a property of a system, whose interfaces are completely understood, to work with other systems without any restricted access or implementation.

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

ITS
Integrated Testing Strategy. An ITS is an approach that integrates different types of toxicological data and information into a decision-making process for the safety of a chemical. In addition to the information from individual assays, test batteries, and/or tiered test schemes, integrated testing strategies may incorporate approaches such as weight-of-evidence and exposure/ population data into the final risk assessment for a substance.

IVIVE
*In Vitro Concentration to In Vivo Dose Extrapolation*

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.

lin-log kinetics
Reaction rates are linearly dependent on enzyme concentration and on the logarithm of concentrations. Rates are defined with respect to a reference state.

Linked Data
A method of publishing structured data, so that it can be interlinked and become more useful. It builds upon standard Web technologies, but rather than using them to serve web pages for human readers, it extends them to share information in a way that can be read automatically by computers. This enables data from different sources to be connected and queried.

Linked Resources
Linked Data approach expanded to all resources including for compounds, biomaterials, assays, algorithms, models, analysis, validation and reports.

LOEL
Lowest Observed Effect Level

LSEC
Liver sinusoidal endothelial cells

LUMO
Lowest Unoccupied Molecular Orbital

MEA
Microelectrode array

Mechanism of toxic action
The mechanism of toxic action is the molecular sequence of events leading from the absorption of an effective dose of a chemical to the production of a specific toxicological response in the target organ or organism.

MeDIP profile
Methylated DNA immuno-precipitation - a method to analyse the DNA methylation across the genome using antibodies directed against modified cytosines (e.g. 5-methylcytosine or 5-hydroxymethylcytosine). Profiling across the genome involved either subsequent next-generation sequencing MeDIP-Seq or array (MeDIP-Chip) technologies.

Meganucleases
Endonucleases, either natural or specifically engineered, that are capable of identifying a very discrete region of the DNA and to cut it, resulting in the disruption of a specific sequence with the potential insertion
of a construct of interest. One construct used in SCR&Tox is a so-called “landing pad”, i.e. a sequence that has been engineered in order to facilitate homologous recombination of various gene constructs that will be secondarily introduced into cells that carry the “landing pad”. Flanking regions of the “landing pad” have been engineered in order to allow meganucleases to retrieve the entire region, leaving no scar in the host genome.

**MID**
Moulded interconnect device

**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) to the estimated exposure dose or concentration.

**Molecular initiating event**
This is the initial point of chemical-biological interaction within the organism that results in a cascade of events leading to an adverse outcome.

**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 landscape across human cells
http://www.roadmapepigenomics.org/

**NMR**
Nuclear magnetic resonance
NOAEC
No observed adverse effect concentration

NOAEL
No observed adverse effect level

NOEL
No observed effect level

Non-testing information
Non-testing data can be generated by three main approaches: a) grouping approaches, which include read-across and chemical category formation; (quantitative) structure-activity relationships ((Q)SARs); and c) expert systems.

NTP
National Toxicological Program

OECD 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.
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.

Polycomb changes
Polycomb proteins are involved in setting and maintenance of epigenetic marks at developmentally regulated genes (such as HOX genes). Changes in the patterns of polycomb genes are indicative of changes in the epigenetic programs set across the genome.

PSCs
Pluripotent stem cells

QC
Quality control

qRT-PCR
Quantitative real-time polymerase chain reaction

QSAR
A Quantitative Structure-Activity Relationship (QSAR) is a quantitative relationship between a biological activity (e.g., toxicity) and one or more molecular descriptors that are used to predict the activity. A molecular descriptor is a structural or physicochemical property of a molecule, or part of a molecule, which specifies a particular characteristic of the molecule and is used as an independent variable in a QSAR.

QT interval
The duration of ventricular depolarization and subsequent repolarization.

REACH
Regulation on Registration, Evaluation, Authorisation and Restriction of Chemicals.

Read-across
A method for filling data gaps in either the analogue or category approaches. Endpoint information for one chemical is used to make a prediction of the endpoint for another chemical, which is considered to be similar in some way. In principle, read-across can be used to assess physicochemical properties, environmental fate and (eco)toxicity effects, and it may be performed in a qualitative or quantitative manner.
In qualitative read-across, the potential of a chemical to exhibit a property is inferred from the established potential of one or more analogues. In quantitative read-across, the numerical value of a property (or potency of an endpoint) of a chemical is inferred from the quantitative data of one or more analogues.

RNA
Ribonucleic acid

RT-CESTM
Real-Time Cell Electronic Sensing

RTD
Research and technical development

SAR
Structure Activity Relationships (SARs) are theoretical models that can be used to predict in a qualitative manner the physicochemical, biological (e.g., toxicological) and fate properties of molecules from knowledge of chemical structure. More specifically, a SAR is a qualitative relationship (i.e., association) between a molecular (sub)structure and the presence or absence of a given biological activity, or the capacity to modulate a biological activity imparted by another substructure. The term substructure refers to an atom, or group of adjacent connected atoms, in a molecule. A substructure associated with the presence of a biological activity is sometimes called a structural alert. A SAR can also be based on the ensemble of steric and electronic features considered necessary to ensure the intermolecular interaction with a specific biological target molecule, which results in the manifestation of a specific biological effect. In this case, the SAR is sometimes called a 3D SAR or pharmacophore.

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).

shRNA
Short hairpin RNA

siRNA
Short interfering RNA

SMARTS
A language in Computational Chemistry for describing molecular patterns.

SOP
Standard Operating Procedure

SQL
Often referred to as “Structured Query Language” is a programming language designed for data management.

Tanimoto criteria
Molecular similarity criteria for chemicals based upon Tanimoto Coefficients.
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.

The ToxBank Chemical Repository will ensure the availability of test compounds to researchers of the SEURAT-1 Research Initiative.

The ToxBank Data Warehouse will establish a centralised compilation of data for systemic toxicity.

The ToxBank Gold Compound Database will provide a information resource servicing the selection and use of test compounds.

**TD**
Toxicodynamics, the processes and interactions of an exogenous compound within an organism, including the compound’s effects on processes at the organ, cellular, and molecular levels.

**TK**
Toxicokinetics, the processes by which a substance reaches its target site. This includes absorption (the process of a substance entering the organism), distribution (the dispersion of substances throughout the fluids and tissues of the organism), metabolism (the irreversible transformation of substances by the organism), and excretion (the elimination of substances from the organism. These four processes are also referred to as ADME.

**Toxicity Pathway**
A toxicity pathway is a cellular response pathway that, when sufficiently perturbed, is expected to result in adverse health effects.

**Toxicological data**
Data relating to the harmful (toxicological) effects of chemicals. This may include information from animal, human or non-animal (*in vitro*) tests.

**TTC**
Thresholds of toxicological concern (TTCs) have been developed for risk assessment of compounds of known chemical structure for which no compound-specific toxicity data are available. Below the TTC value the risk to human health is assumed to be negligible. The TTC may be used as a substitute for substance-specific information in situations where there is limited or no information on the toxicity of a compound, and where human exposure is so low, i.e. below the corresponding TTC, that adverse effects are not to be expected.

**US FDA**
United States Food and Drug Administration

**US EPA**
United States Environmental Protection Agency

**VE-cadherin**
Vascular endothelial cadherin

**Web Service**
A method of communication between two electronic devices over a network.

**ZFN-HR**
Zinc finger nuclease homologous recombination.
Nicola Diane Douglas
(1952-2012)

Nicola Diane Douglas, known to her family, friends and co-workers as Nicki, was born 27 March 1952 in Barton-on-sea in the UK, the daughter of Charles and Diana Hasemer. She died suddenly and unexpectedly in her home in Zeiningen, Switzerland on 6 February 2012.

Due to her father’s work as an airline pilot, she travelled extensively in her youth. She maintained an interest in travel and global issues throughout her life, particularly in the area of animal protection and wildlife conservation. This interest was put into action through initiation and support of research in the development of alternatives to replace animal testing, involvement in activities in Switzerland on animal protection, and participation in wildlife conservation work in Africa.

Her early professional life involved photography, journalism, and teaching English as a foreign language, after which she settled down into marketing and communications, following her move to Switzerland in 1975. As part of the country management team in Switzerland for Autodesk (creators of AutoCAD), she was responsible for all marketing activities in multiple languages throughout Europe and the Middle East and worked closely with the company’s virtual corporation of third-party developers.

Nicki Douglas and Barry Hardy met in August 1999 and lived and worked closely together as partners in Switzerland from September 2001 until her death in 2012.

In 2003 she founded Douglas Connect (DC) as a Swiss SME which specialised in creating and facilitating research communities and networks, collaborative projects and knowledge management. DC created and directed the program activities of the InnovationWell and eCheminfo communities of practice with goals and activities aimed at improving human health and safety and developing new solutions for neglected diseases. DC served as Coordinator of the FP7-supported OpenTox project (www.opentox.org) which developed an Open Source Predictive Toxicology Framework for the management of toxicology data, models and validation. Within SEURAT-1, DC is the current scientific coordinator of the ToxBank infrastructure development project for supporting predictive toxicology and risk assessment (www.toxbank.net). DC is also leading research activities in antimalarial drug design and toxicology for the Scientists Against Malaria project (www.scientistsagainstmalaria.net) which was developed from a pilot within the SYNERGY FP7 ICT project on knowledge-oriented collaboration. In addition to managing the business, Nicki participated hands-on in communications with many scientists involved in the communities and collaborations, and organized numerous meeting and workshop activities, whose success was due to her attention to detail and the social interaction cultivated.

In recent years Nicki and Barry have been active in wildlife conservation work in southern Africa including work in Namibia and South Africa with goals to sustain and support wilderness areas and their populations of wildlife. They have been involved in work aimed to develop a new park in Namibia, and participated in lion capture, leopard tracking and African wild dog monitoring. Nicki in particular had a special affection and communication with cats, both domestic and wild. It is planned to establish a foundation in Switzerland which will continue to support similar research and conservation activities in the future.

Barry Hardy,
Scientific Coordinator of ToxBank
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.
The seventh amendment of the „Council Directive on the approximation of the laws of the Member States relating to cosmetic products” (76/768/EEC) foresees a deadline (2013) for the replacement of animal testing of cosmetic products in the fields of repeated dose toxicity, reproductive toxicity and toxicokinetics. The European Commission together with Cosmetics Europe launched a Research Initiative entitled „Towards the replacement of in vivo repeated dose systemic toxicity testing” in order to develop a innovative research strategy leading to the long-term target „Safety Evaluation Ultimately Replacing Animal Testing” (SEURAT). This Research Initiative is called SEURAT-1 and comprises six integrated projects focusing on the development of new non-animal test methods in the field of repeated dose systemic toxicity.

This is the second volume out of a series of six annual books that will, step by step, pave the way towards innovative safety evaluation of chemicals in various fields of application (e.g., medicine, personal care, agriculture, food production, ingredients of everyday products).

The specific goal of this Research Initiative is the development of in silico and in vitro test systems based on cell lineages derived from human stem cells, which is considered to be a first step towards 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 policy makers about scientific progress relevant to the implementation of European Directives and Regulations, fully respecting the 3Rs-principle
- to inform the research policy makers 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 increasing their competitiveness
- to encourage the extension of the Research Initiative activities on National, European and International levels