EUROPEAN COMMISSION
Research & Innovation

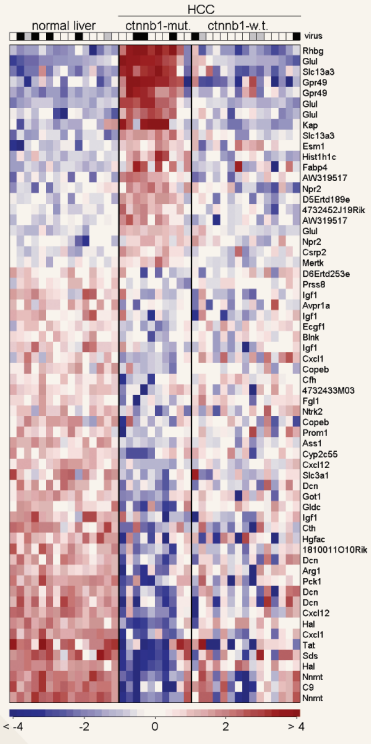
COLIPA
The European Cosmetics Association

SEURAT-1

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

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

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



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Paracelsus (1493 - 1541)
Portrait by Quentin Massys

« The dose makes the poison »

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

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

This is the first 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"

Launch of the European Research Initiative on human safety assessment

Edited by:
Michael Schwarz, Tilman Gocht

volume 1

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

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

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Foreword

Nearly 100 scientists from over 70 European organisations are working together in a EUR 50 million research and innovation project in the field of novel human safety testing solutions. This unique collaboration is possible thanks to the European Union's Health Programme and an initiative of the European cosmetics industry.

This Research Initiative was launched in July 2009 under the 7th framework programme for Research and Development (FP7) by the European Commission. The European cosmetics industry offered to match the European Commission's funds to make a total available of EUR 50 million to try to fill current gaps in scientific knowledge and accelerate the development of non-animal test methods. The initiative focuses on the complex area of repeated dose toxicity.

The Research Initiative is a first step to addressing the long term strategic target of 'Safety Evaluation Ultimately Replacing Animal Testing (SEURAT)'. It is called '**SEURAT-1**', indicating that more steps have to be taken before the final goal will be reached. **SEURAT-1** will develop knowledge and technology building blocks required for the development of solutions for the replacement of current repeated dose systemic toxicity testing *in vivo*, used for the assessment of human safety. The **SEURAT-1** Research Initiative started on 1 January 2011 and will run for five years.

Cosmetic products and ingredients launched on the European market need to be safety assessed for human health. For many years, substantial efforts have been made by public research programmes and by the cosmetics industry to develop alternative and more efficient solutions to the *in vivo* toxicity tests used for assessing human safety of new products and product ingredients. However, the current state of knowledge still does not allow complete replacement of animal testing, especially as regards the effects of products and ingredients used repeatedly over longer periods (repeated dose effects).

The development of non-animal alternative methods represents a considerable scientific challenge. The cosmetics industry has been working hard for many years to meet this challenge as fast as possible. The development, validation and acceptance of alternative methods by regulatory bodies can only be considered as a long-term effort. Therefore, in addition to the research and technology development work, the **SEURAT-1** Research Initiative will also elaborate a longer-term roadmap ensuring that European research efforts can be united, and invested efforts optimised, in order to achieve deployable solutions as soon as possible.

Bertil Heerink,

Director General of Colipa (The European Cosmetics Association)

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Executive Summary

This publication is the first volume of a series of six Annual Reports that summarises the activities of a new 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's Directorate General for Research and Technology Development (DG-RTD) within the HEALTH theme of the Seventh European Research Programme (FP7) and the European Cosmetics Association (Colipa). The framework for this Research Initiative was created through a call for proposals within the FP7 in June 2009: 'Alternative Testing Strategies: Towards the replacement of *in vivo* repeated dose systemic toxicity testing' with a total budget 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 HEALTH theme of the European Commissions's DG-RTD in 2008. It is called **"SEURAT-1"**, indicating that this is a 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 concept and corresponding long-term research strategy for future 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. By this, **SEURAT-1** will contribute to developing a new definition of 'adversity' in toxicology at the cellular and molecular level leading to pathway-based human safety assessment. This will require the establishment of a complex system consisting of stable human cell lines and organ-simulating devices in combination with computational chemistry, systems biology and sophisticated modelling and estimation techniques. Emphasis will be put on the reliability and reproducibility of all components necessary for the implementation of the concept.

Key elements of the SEURAT-1 Research Initiative
Hypothesis driven approach to elucidating modes-of-action and identifying associated key events and biomarkers.
Emphasis on *in vitro* models that capture important modes-of-action directly relevant to human physiology. Exploit stem cell technology to develop *in vitro* systems with cellular diversity to model higher level functions.
Development of fit-for-purpose in vitro assays suitable for High Throughput Screening (HTS) implementation. Use of bioreactors to engineer tissue comprising multiple cell types to model complex toxicological processes.
Biokinetic modelling to extrapolate between *in vitro* test concentrations and repeated dose organ exposure *in vivo*. Computational toxicology to associate chemicals with molecular initiating events and describe metabolism
Use of high content analysis tools including 'omics to describe modes-of-action at the molecular level. Systems biology approaches to model mode-of-action dynamics at the molecular scale for quantitative analysis.
Proof-of-concept exercise to demonstrate a mode-of-action based integrated test system to predict sub-chronic liver toxicity Feasibility study to show how test data can be used in a safety assessment context

To achieve this goal, a cluster of projects has been organised under the umbrella of the **SEURAT-1** Research Initiative. The cluster is composed of six integrated projects, which will run for five years. These projects will closely cooperate and combine the research efforts of over 70 European universities, public research institutes and private companies. The collaboration between these six integrated projects, the dissemination of results, the cooperation with other international research teams, and the continuous updating on research priorities will be facilitated by a separate coordination and support action project (referred to as COACH). These collaboration activities are strongly supported by the Scientific Expert Panel, which in particular plays a key role in providing scientific advice regarding the research work and future orientation of the **SEURAT-1** Research Initiative.

This first Annual Report, prepared by the coordination and support action project COACH, presents a comprehensive overview of the work in the different projects of the **SEURAT-1** Research Initiative. This is given in the context of recent developments in European legislation regarding 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, as well as the objectives and the structure of **SEURAT-1**.

Chapter 2 presents the context of the **SEURAT-1** Research Initiative from various perspectives. (i) Legislation: The EU Cosmetics legislation currently foresees a deadline in 2013 for the replacement of animal testing of substances used to formulate cosmetic products for the properties of repeated dose toxicity, reproductive toxicity and toxicokinetics. However, a panel of experts appointed by the European Commission recently came to the conclusion that no clear timeline for full replacement of animal testing methods can be given for repeated dose toxicity due to the underlying scientific challenges. Besides the EU 'Cosmetics Directive', the **SEURAT-1** Research Initiative is also relevant for other important EU Regulations such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) and CLP (Classification, Labelling and Packaging of substances and mixtures). (ii) Science: Toxic responses seen *in vivo* after repeated dosing are often different to effects following acute exposure due to different mechanisms of action, as discussed in more detail in this chapter. Hence, the development of animal-free *in vitro* methods to assess repeated dose toxicity requires the identification of the related underlying molecular mechanisms.

Chapter 3 focuses on the development of a long-term research strategy and describes the main elements of the research programme to be undertaken over the course of **SEURAT-1**. The strategy is to adopt a toxicological mode-of-action framework to describe how a substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that together as an integrated package predict long-term general toxic effects as quantitative points of departure that are needed for safety assessment. The final output of the **SEURAT-1** Research Initiative will be to deliver a proof-of-concept showing how the developed scientific tools and knowhow can be combined to create animal-free decision support systems for human safety assessment.

The detailed project descriptions 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 are:

ScrTox	Stem cell differentiation for providing human-based organ specific target cells to assay toxicity pathways *in vitro*
HeMiBio	Development of a hepatic microfluidic bioreactor mimicking the complex structure and function of the human liver
DETECTIVE Detection of Endpoints and Biomarkers for Repeated Dose Toxicity Using In Vitro Systems	Identification and investigation of human biomarkers in cellular models for repeated dose *in vitro* testing
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
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
ToxBank	Data management, cell and tissue banking, selection of reference compounds and chemical repository
COACH	Cluster level coordinating and support action

This chapter also contains reports about the kick-off 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 are discussed as well.

Chapter 5 describes the expected outcomes of the **SEURAT-1** Research Initiative at the end of the 5 years in the context of related international activities. For the success of **SEURAT-1** 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 for better human safety assessment of chemicals for the future.

Unique elements in the structure of the SEURAT-1 Research Initiative

- Funding scheme: Joint funding by the European Commission and the European Cosmetics Association (Colipa).
- Coordinated cluster of RTD projects: Installation of a Coordinating Action right from the start; with its coordination mechanisms it will provide a maximum of synergy between participants and therefore optimise the output of this cluster of projects
- Data management: Installation of a data management and servicing project right from the start

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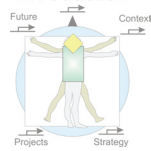

1 INTRODUCTION

„Most of the toxicology tools used for regulatory assessment rely on high-dose animal studies and default extrapolation procedures and have remained relatively unchanged for decades, despite the scientific revolutions of the past half-century."

Hamburg[1], M.A., 2011: Advancing Regulatory Science. Editorial.- Science, 337, pp. 987.

[1] *Margaret A. Hamburg is Commissioner of the U.S. Food and Drug Administration.*

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, the European Cosmetics Association (Colipa) offered at the beginning of 2008 to the European Commission the contribution of EUR 25 million in order to fund 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. Colipa 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 is called '**SEURAT-1**', indicating 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. Colipa 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 Colipa.

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

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

The **SEURAT-1** Research Initiative has started in January 2011. It is the first time the issue of alternatives to using animals for repeated dose systemic toxicity testing is addressed in EU-funded projects. It should be noted that only proposals that do not themselves involve tests on living animals were eligible for funding.

Even though the **SEURAT-1** Research Initiative was initially motivated by the cosmetic indus-

try, it is of course 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 which should be 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 project 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 following research programmes. This includes establishing innovative scientific tools required for better understanding of repeated dose toxicity based on *in vitro* tests, and identify gaps of knowledge 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.

The objectives of the **SEURAT-1** Research Initiative are

- to produce and use human-based, stable cell lineages based on stem cell differentiation
- to consider modulations in toxicological pathways through cell-cell interactions by means of artificial, organotypic cell systems
- to assess epigenetic effects *in vitro*
- to explore biomarkers for the respective toxicological pathways
- to convert *in vitro* results into *in vivo* predictions
- to develop prediction models based on mechanistic process understanding for future safety evaluation of chemicals *in silico*

The research work will include 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. Taking into account the complexity of the problems to be solved and the broadness of expertise needed to address the underlying scientific questions, a coordination of efforts between academia, industry, regulators and other stakeholders on national, European and also international level is needed.

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. This structure is illustrated in *Figure 1.1*.

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*

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

➠ 'Hepatic Microfluidic Bioreactor' (*HeMiBio*): Development of a hepatic microfluidic bioreactor mimicking the complex structure and function of the human liver

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

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): 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

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): 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

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

➠ '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

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): Cluster level coordinating and support action

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 for the cluster is provided either directly through the Scientific Secretariat, or through the SEP.

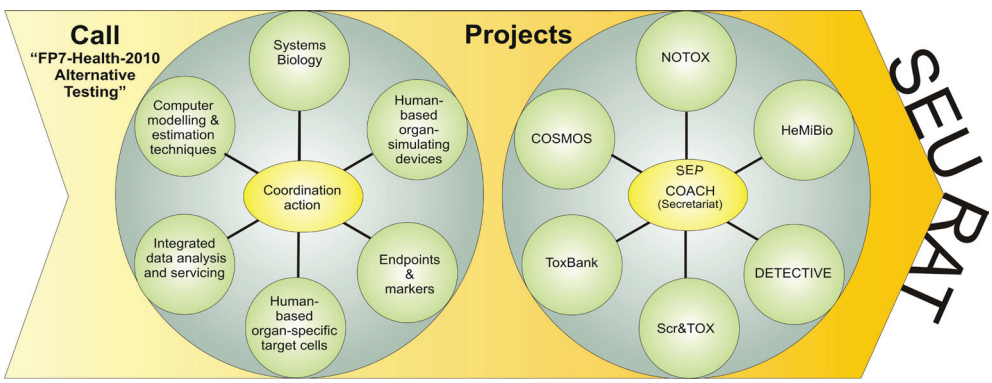

Figure 1.1 *Building blocks of the* ***SEURAT-1*** *Research Initiative that were established based on the call for proposals under the HEALTH Theme of the 7th European RTD Framework Programme 'Towards the Replacement of in vivo Repeated Dose Systemic Toxicity Testing'.* ***SEURAT-1*** *is supposed to be the first step in a specific area addressing the global long-term strategic target SEURAT ('Safety Evaluation Ultimately Replacing Animal Testing').*

The Annual Report: Something about ‘Pathways’

This is the first volume of a series of six Annual Reports that will present a comprehensive overview of the planned work in the different projects of the **SEURAT-1** Research Initiative. The following volumes will then focus on major results obtained in the research projects and of progress made towards reaching the final goal of the cluster. It is intended that the structure of the Annual Reports will be kept over the six-year period in order to facilitate progress monitoring. Hence, 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: The structure of the Annual Report is inspired by one of the most important keywords of the addressed field of research, which is “toxicity pathways” (*Figure 1.2*). Conceptual considerations related to biological pathways leading to toxicity will consistently guide through the report series. Overall, 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. But the analogy is even stronger:

Chapter 2 describes the context of the **SEURAT-1** Research Initiative as a whole. The context of a toxicity pathway is given by the functionality of a cell, an organ, or the entire organism. The context of the **SEURAT-1** Research Initiative is given by legislation and policy with respect to the regulation of chemicals (i.e. safety assessment), that will be described in chapter 2. Over the course of **SEURAT-1**, this chapter will provide insight about how legislation and policy adopt scientific progress in the field of repeated dose systemic toxicity. Furthermore, the scientific state-of-the-art will be described in detail with special emphasis on current test methods and mechanistic aspects in toxicology.

The following chapter 3 will outline the long-term research strategy of the SEURAT initiative (i.e. **SEURAT-1** and beyond), and describes an execution plan that outlines the main elements of the research programme to be undertaken. In this context the main research priorities of the first execution phase, **SEURAT-1**, are described and it is proposed how the results and momentum can be carried forward to the next phase, **SEURAT-2**. Again, the analogy to “toxicity pathways” is obvious, as a toxicological pathway follows a sequence of events. Similarly, **SEURAT-1** follows a strategy that will lead research in a distinct direction, namely how the research programme should be organised to get insights into the mechanisms involved in repeated dose systemic toxicity, and, more specifically, how *in vitro* test systems must be designed in order to fulfil the requirements for safety evaluation of chemicals. The research strategy provides the main scientific questions to be answered through the cluster projects. On the other hand, the research strategy will be iteratively refined through the data generated in these cluster projects. Thus, the refinement of the research strategy is the overarching goal integrating the different cluster projects into one concept.

This chapter is followed by the detailed project descriptions in chapter 4 that provides an overview about the specific scientific challenges, methods how to meet them and expected results. Like a toxicological pathway consists of single events, this **SEURAT-1** Research Initiative consists of individual elements that, altogether, are systematically connected. These interactions between the projects, as well as the planned activities within the first year are also presented here.

Finally, a pathway has an endpoint, which is an adverse effect at the end of a toxicological pathway. At this point the analogy with the structure of the Annual Report does not work any longer as the final chapter 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.

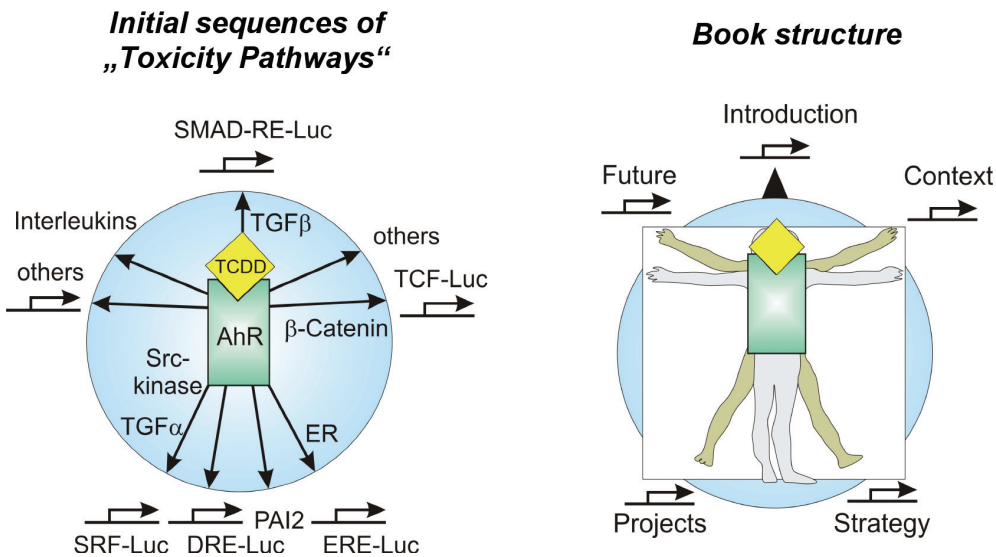

Figure 1.2 *The concept of primary molecular events triggering „Toxicity Pathways“ (left panel) is mirrored by the book structure (right panel).*

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 closely work together on common scientific objectives. The participation of SMEs in **SEURAT-1** is high with more than 30%.

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

Participant	Institution	Project
Project Coordinators		
Marc Peschanski	INSERM/UEVE 861, I-STEM/AFM, Evry /France	SCR&TOX
Catherine Verfaillie	Interdepartmental Stem Cell Institute, Katholieke Universiteit Leuven / Belgium	HEMIBIO
Jürgen Hescheler	Institute for Neurophysiology, University Hospital Cologne / Germany	DETECTIVE
Mark Cronin	School of Pharmacy and Chemistry, Liverpool John Moores University / UK	COSMOS
Elmar Heinzle	Biochemical Engineering, Saarland University, Saarbrücken / Germany	NOTOX
Barry Hardy	Douglas Connect, Zeiningen / Switzerland	TOXBANK
External Experts		
Roger Arnold Pedersen	Laboratory for Regenerative Medicine and Cambridge Stem Cell Initiative, University of Cambridge / UK	
Hans Juergen Ahr	Bayer Health Care AG, Wuppertal / Germany	
Emanuela Testai	National Institute for Health, Dept. of Environment and Primary Prevention - Mechanism of Toxicity Unit, Rome / Italy	
Gabrielle Hawksworth	Division of Applied Medicine, University of Aberdeen / UK	
Ian Cotgreave	AstraZeneca Safety Assessment, Södertälje / Sweden	
Catherine Mahony	Colipa (Procter & Gamble), London Innovation Centre / UK	
Derek Knight	European Chemicals Agency, Helsinki / Finland	

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

2 CONTEXT

"The 3Rs principle is, for the first time, explicitly described and firmly established in EU legislation. Strict provisions will be brought in on how the 3Rs are to be implemented, and these will be applied to all sectors using animals under their own, specific legislation, where it exists."

J. Potočnik, European Commissioner for Environment. In: EPAA Newsletter, March 2011.

2.1 Introduction

The 7th Amendment to the Cosmetics Directive introduced a number of key requirements relating to animal testing, which have been incorporated into the Cosmetics Regulation (Regulation (EC) No 1223/2009, 30 November 2009. Already in 2004 a ban was introduced on the testing of cosmetics products on animals within the EU. In 2009 an EU testing ban of cosmetic ingredients came into force with an extension till 11 March 2013 for three specific areas (repeated dose toxicity (includes skin sensitisation, carcinogenicity and subacute/subchronic toxicity), reproductive toxicity (also includes teratogenicity) and toxicokinetics).

The 2013 regulatory deadline prompted a joined effort from the Directorate General of Research and Innovation of the European Commission and the European Cosmetics Association (Colipa) to launch the Research Initiative 'Towards the replacement of *in vivo* repeated dose systemic toxicity testing'. It is called '**SEURAT-1**', indicating that this is a first step in a specific area addressing the long-term strategic target SEURAT, which represents the long-term target 'Safety Evaluation Ultimately Replacing Animal Testing'.

This chapter highlights the recent developments in legislation regarding chemicals with special emphasis on cosmetic ingredients. Furthermore, it introduces the specific field of repeated dose toxicity and presents an overview about state-of-the art methods for chemical testing considering repeated dose exposure scenarios.

2.2 Requirements for Risk Assessment: the Perspective of Authorities in the Context of REACH and CLP

Derek J. Knight

Cosmetic ingredients are chemical substances that are subject to the EU REACH and CLP Registrations, as well as the EU Cosmetics Regulation. REACH stands for Registration, Evaluation, Authorisation and Restriction of Chemicals, and the Regulation is (EC) No 1907/2006 (*Anonymous, 2006*). CLP is the Classification, Labelling and Packaging Regulation, i.e. (EC) No 1272/2008 (*Anonymous, 2008*). This means that the cosmetic ingredients require a REACH registration (unless otherwise exempt) and industry has to make sure they are

manufactured and used safely in terms of effects on health and the environment for all EU life-cycle stages. Hence the Chemical Safety Report (CSR) has to cover full human health assessment for substances classified as dangerous, except for the life-cycle stage of use of the cosmetic ingredient in finished cosmetic products because assessing this risk is covered by obligations under the Cosmetics Regulation. Furthermore, CLP applies to cosmetic ingredients, whether neat or as components of chemicals mixtures, in the EU, except when in the finished cosmetic product. Hence **SEURAT-1** is directly relevant to the cosmetics industry both in the context of EU chemical substance regulations and the cosmetics regulation.

The overall purpose of both the REACH and the CLP Regulations is to ensure a high level of protection of human health and the environment. Industry has to ensure that chemical substances are used safely. This is achieved by using information on the properties of substances to assess their hazards both for classification and risk assessment, and hence to develop appropriate risk management measures to protect human health and the environment. One of the main reasons for developing and adopting the REACH Regulation was to fill information gaps for the large number of substances already in use in the EU, as for many there is inadequate information on their hazards and risks they pose. REACH prescribes that, in general, all substances manufactured or imported in quantities at 1 tonne or more per year in the EU have to be registered.

REACH registrants have to provide information on the intrinsic properties of the substance in the registration dossier. The standard information required depends on the tonnage manufactured or imported; the higher the tonnage, the more information needed. In addition for substances at 10 tonnes per annum or above, the registration dossier must include a CSR. A repeated dose toxicity study is part of the standard information requirements for substances registered at >10 tonnes per annum, but there are circumstances under which the registrant can instead include this study in a Testing Proposal for the study to be conducted after registration, providing adequate risk management measures are taken in the meanwhile to take account of the delay in obtaining information on the repeated dose effects in humans.

The CLP Regulation does not require new studies to be conducted, although some suppliers may choose to do this. Industry has to obtain all the available relevant information and evaluate it in terms of the CLP classification criteria in order to classify their chemical substances and mixtures for hazard communication in terms of labelling, providing Safety Data Sheets (SDSs) and using suitable packaging (*ECHA, 2011*). In practice this means that many substances can be classified on the basis of the data obtained during the preparations for registration under REACH.

New studies using vertebrate animals for REACH registration should only be conducted as a last resort. In addition there are data sharing obligations for registrants of the same substance to avoid duplicate testing using experimental animals. Registrants must first collect

and assess all existing data. Then they have to identify data gaps and consider whether they can be filled by using non-standard data before any new tests are conducted. This means that all available information is collected: *in vivo* and *in vitro* studies, information from human exposure, information from structurally-related substances (i.e. 'read-across' and 'chemical categories') and predictions from valid (Q)SARs. There are provisions in REACH for using non-standard information in Annex XI that explains the principles of 'adapting' the standard information requirements (these are listed in the 'data' Annexes VII to X) and there is detailed ECHA guidance and manuals on this (*ECHA, 2009*, *2010a, b, c, d, e, f)*. The non-standard information has to be equivalent to the information obtained from the standard test data. The key point is that the non-standard data must be suitable for an adequate risk assessment to ensure the substance can be used safely and also for adequate classification for hazard communication. Registrants have to justify these adaptations of the standard information requirements in the registration dossier and provide scientific explanations why the non-standard data is nevertheless adequate. Within this context is should be noted that industry remains responsible for assessing the intrinsic properties for hazard and/or risk assessment and classification; hence they are responsible for making the technical and scientific judgments. However, ECHA can require missing information to be provided, including tests if the data waivers or non-standard data do not meet the information needed for registration, as an outcome of the dossier evaluation processes.

Furthermore it should be remembered that in conducting a risk assessment for human health the derived no effect level (DNEL) concept is applied for the CSR. The set of information relating to effects on humans is used to derive a dose at which it is expected that no adverse effects will occur in humans for that particular exposure route and duration. The results from animal toxicology studies are used to model effects in humans by applying assessment factors to take account in uncertainty in using data from animal studies to predict effects in humans. There are 'default' standard assessment factors recommended in the ECHA guidance, but registrants can deviate from these on a case-by-case basis to take account either of extra uncertainty from the data set or of a lower uncertainty (e.g. if epidemiology data are available or toxicokinetics or dynamic data indicate difference between animals and humans). There must be an objective basis to such adjustments of the standard assessment factors, and they should be explained and justified in the CSR. Therefore, if a registrant uses non-standard data to derive a DNEL he may need to adjust the assessment factor to take account of extra uncertainty in the hazard assessment. It is of the utmost importance to understand that the registrant is responsible for assessing the risk in order to ensure that adequate risk management measures are in place to control the risks. Therefore to discharge this duty he has to be satisfied that the registration data enable him to undertake an adequate risk assessment. In practice registrants will use professional judgement and apply good science in assessing the properties of substances and assessing the risks from their uses.

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2.3 Positioning of the SEURAT-1 Research Initiative in Relation to the Cosmetics Directive

Rob Taalman

2.3.1 Regulatory Challenges

The Cosmetics Directive 76/768/EEC, provides the European regulatory framework for the placing on the market of cosmetic products. The 7th Amendment to the Cosmetics Directive introduced a number of key requirements relating to animal testing, which have now been incorporated into the Cosmetics Regulation (Regulation (EC) No 1223/2009, 30 November 2009. A ban on animal testing of finished cosmetic products has been in force since September 2004 and a testing ban on ingredients or combinations of ingredients since March 2009. As from March 2009, it is also prohibited in the EU to market cosmetic products and their ingredients which have been tested on animals, irrespective of the origin of these products. There is an exemption for the most complex human health effects - these being repeated dose toxicity, including skin sensitisation and carcinogenicity, reproductive toxicity and toxicokinetics - for which the deadline is extended to March 2013.

This year (2011), the European Commission will review the situation regarding the technical feasibility of complying with the 2013 ban and inform the European Parliament and the Council, proposing measures to be taken if necessary. In this context the European Commission appointed a panel of experts in 2010 to review the current status and future prospects of alternative (non-animal) methods for cosmetics testing, and to provide realistic estimates of the time required for the development of alternative methods where not already existing. The expert panel report was subject to public consultation prior to its finalization and publication (*Adler et al., 2011*). The European Centre for the Validation of Alternative Methods (ECVAM), hosted by the Institute for Health and Consumer Protection of the European Commission's Joint Research Centre, coordinated the whole review activity on behalf of the Commission's Directorate General for Health and Consumers.

2.3.2 State of the Science - Non-Animal Methods (2010)

The experts concluded that significant progress has been made in reducing the number of animals used in tests via the use of *in vitro* tests and computer-based modelling alongside animal tests but considerable scientific challenges remain and full replacement of animal tests is not yet possible. The expert report predicts that, for the five specific areas identified, full replacement alternative testing methods will **not** be available by 2013.

More specific:

> ➠ No clear timeline could be given for the areas of **toxicokinetics**, **repeated dose toxicity**, **carcinogenicity and reproductive toxicity** due to the underlying scientific challenges.
>
> ➠ The timelines estimated for full replacement of animal tests in the area of **skin sensitisation** point to a further 7-9 years (i.e. 2017-2019), including the possibility to differentiate weaker from stronger sensitisers. Alternative methods able to simply discriminate between skin sensitisers and non-sensitisers might become available earlier. Please note that skin sensitisation is not part of the **SEURAT-1** Research Initiative.

In this regard, the forecasts for the full availability of alternative test methods do not diverge much from estimates provided in a similar review already conducted by the Commission in 2005 (*Eskes and Zuang, 2005*). Even though some progress has been made since then, full replacement of animal testing will require continuous international research efforts and scientific exchange between related research programmes (such as the **SEURAT-1** Research Initiative and the US activities in the field, see chapter 5.3 and *Spielmann et al., 2010*).

2.3.3 Future Prospects

The 2010 expert report underlines the continuous effort both in Europe and worldwide to find alternative approaches that avoid testing on animals wherever possible. As a result of this effort, understanding of toxicological processes in the human body has improved significantly over the last decade, and continues to do so at an accelerating rate. Advanced methods and approaches hold a lot of promise for the future development of more predictive risk assessment, based on improved understanding of how toxic substances reach the target cells/organs (toxicokinetics) and perturb critical biological pathways. International cooperation and collaboration has never been as extensive in this field as now and shared access to an increasing amount of data and tools will allow a new generation of test methods and integrated test systems to emerge. The descriptions of both *in vitro* and computational models in the tables and accompanying texts of the full report illustrate the many alternative methods under current development. The central importance of toxicokinetics is underlined.

The report highlights that the current momentum for developing alternative methods and testing strategies should be maintained. Research and development activities in the field of non-animal testing, both in the public sector (European framework programmes and national research programmes) and industry, have already yielded many promising methods and approaches, and these activities including the joint effort of the EC and the Cosmetics industry association in the context of the **SEURAT-1** Research Initiative should be further stimulated and encouraged.

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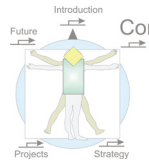

2.4 Introduction into Repeated Dose (Systemic) Toxicity

Michael Schwarz, Catherine Mahony

2.4.1 Repeated Dose (Systemic) Toxicity: *A* definition

The following definition of repeated dose toxicity is taken from a recent publication (*Adler et al., 2011*).

"The term repeated dose toxicity comprises the general toxicological effects occurring as a result of repeated daily dosing with, or exposure to, a substance for a part of the expected lifespan (sub-chronic exposure) or, in case of chronic exposure, for the major part of the lifespan of the experimental animal. The onset and progression of this toxicity is influenced by the interplay between different cell types, tissues and organs, including the concomitant contribution of toxicokinetics, hormonal effects, autonomic nervous system, immunosystem and other complex systems. Current repeated dose toxicity studies provide information on a wide range of endpoints because changes in many organs and tissues are taken into account. They allow evaluation of an integrated response and its quantitative (dose–response) aspects, making its replacement very challenging."

Toxic responses seen after repeated dosing can be local (e.g. after dermal exposure to a chemical) or systemic, affecting internal organs. **SEURAT-1** will iteratively develop an innovative concept for repeated dose systemic toxicity testing. The goal is to demonstrate that this concept constitutes a solid basis for a future full implementation of the new toxicity pathway strategy.

2.4.2 Repeated Dose Systemic Toxicity as Opposed to *A*cute Toxicity: Some Mechanistic Considerations

The effects produced by chemical agents including drugs may strongly differ between acute and (sub)chronic exposure.

Acetaminophen (Paracetamol), taken as a first example, is a widely used and generally well-tolerated analgesic and antipyretic drug. The compound is metabolised, mainly in the liver, to a toxic metabolite N-acetyl-p-benzo-quinone imine (NAPQI) which, if not detoxified, may potentially interfere with cellular functions by binding to cellular macromolecules and inducing the formation of reactive oxygen species and lipid peroxidation. At therapeutic doses, however, the toxic intermediate formed is completely detoxified within the hepatocytes by glutathione conjugation. Only at high doses, when the glutathione pool becomes depleted, does the toxic intermediate accumulate within the liver cells that produce it and will eventually kill them

(*Graham et al., 2005*). In other words, a single very high dose will produce acute liver failure because of massive necrotic hepatocyte death but the same total amount of compound split into smaller daily doses can repeatedly given without generating the unwanted toxic side effect.

This is not always so. In the case of the so called “ct”-rule, a toxicological effect is the result of total dose over a period of time, such that even very small doses, given for prolonged periods of time, will produce the same or a similar toxic effect as a high dose given for only a short period of time. The product of concentration (c) and exposure time (t) is constant for this type of poisons (c x t = const.). Haber (1868-1934) developed this rule while studying the acute toxicity of war gases and the principle is well-accepted in occupational risk assessment, where the use of time-weighted-averages (TWAs) are routinely used. The principle behind Haber’s rule is also well-accepted in non-cancer risk assessment, where risk estimates based on subchronic toxicity studies are adjusted by an uncertainty factor in the range of 3-10-fold to extrapolate to a potential chronic/lifetime human exposure (i.e., a higher exposure for a shorter duration of time is toxicologically equivalent to a lower exposure for a longer period of time). The range of uncertainty factor from 3 – 10 is based on an evaluation of empirical data but it is noted that this adjustment is also in the range of what would be predicted by applying Haber’s Rule. Specifically, a subchronic study of 90 days duration is taken to be ~ 10% of an animals’ lifetime, so that theoretically, an exposure of x mg/kg/day for 90 days would be toxicologically equivalent to an exposure of 0.1x for a lifetime. It is noted that many of the analyses based on empirical data find the default 10-fold uncertainty factor to be quite conservative, with the mean/median values tending to be more in the range of 2 to 5-fold.

Haber’s Rule has been applied in the area of cancer risk assessment in a more direct fashion. For a genotoxic carcinogen, it is assumed that a molecule at the right time and place can interact with DNA resulting in the initiation of a stage in the carcinogenic process. If this is a stochastic event, the number of such occurrences is proportional to the total number of opportunities. Very few molecules may interact with DNA and very few of these reactions may proceed through all stages to a tumor, but the probability of a tumor is proportional to the total number of molecules available (total dose).

The mutagenic activity of genotoxic carcinogens enhances the probability of cancer development even at very low but repeated exposures. Fortunately, there are now quite a few *in vitro* assay systems available, that are able to detect mutagenic effects of chemicals. As a consequence, demonstration of mutagenicity will normally stop progression of a drug or a cosmetic ingredient very early during its development. Only in specific cases, such as with certain anti-cancer drugs, where the benefits clearly outweigh the unwanted side effect of an increase in the risk of secondary cancer formation, will such drugs make it to the market. Agents with proven mutagenic activity are not in the center of interest of the **SEURAT-1** Research Initiative research, however, simply because there already exist methods that allow the detection of this kind of activity

The toxicological testing in experimental animals for adverse effects of chemical agents in humans routinely includes repeated dosing scenarios, often for 28 or 90 days, or even life-long exposure. The reason for this is that chemical agents may produce adverse effects in the experimental animals upon repeated dosing which differ in nature from those seen upon single acute exposure.

A good example is phenobarbital. This barbiturate is used as an anti-epileptic drug both in human and veterinary medicine. In rodents, the compound is often used as a model tumor promoter which increases the risk of liver cancer in exposed animals when given subsequent to a tumor-initiating carcinogen (*Moennikes et al., 2000*). The available evidence, however, questions the relevance of this effect for humans since a significant increase in the incidence of primary liver cancer could not be demonstrated in several large epidemiological studies. In experimental animals (e.g. mice and rats) the drug causes liver enlargement and an increase in the level and activity of a variety of drug-metabolizing enzymes, including some isoforms of cytochrome P-450. This effect is seen even after a singly high dose and is fully reversible. Upon continuous exposure liver enlargement and enzyme induction persist but are completely reversed to normal after cessation of treatment. However, when given to rats and mice at sufficiently high doses and for sufficiently long periods of time phenobarbital will also increase the risk of liver cancer, an effect that is only partly – if at all - reversible after cessation of treatment. This latter activity is not caused by a genotoxic activity – phenobarbital is not mutagenic – but by a tumor promotional effect of the barbiturate. It is known that all these effects are mediated through activation of a nuclear receptor, the constitutive androstane receptor (CAR), and are therefore absent in CAR-knockout mice (*Yamamoto et al., 2004*). Recent observations made by Jonathan Moggs and colleagues in the Innovative Medicine Initiative (IMI) project MARCAR have shown that exposure of mice to phenobarbital for several weeks causes a change in the liver DNA methylome, an effect not seen after only one exposure. This change in locus-specific DNA methylation pattern, which is only seen after prolonged periods of phenobarbital treatment, will then cause a change in the expression of certain RNAs which may trigger the tumor promotional effect of the barbiturate seen after chronic treatment for weeks or months but not seen after acute exposure which only leads to the adaptive and fully reversible liver growth and enzyme induction response aimed to facilitate elimination of the drug from the body. Future research will show whether long-term toxicity mediated by drug-caused specific changes in the target cells methylome can be predicted from cellular systems where the target cells, in this case the hepatocytes, have been differentiated *in vitro* from stem cells.

A second good example is carbon tetrachloride. The chemical is a strong liver toxicant since it is metabolically activated in hepatocytes to a radical intermediate which can covalently bind to proteins and induce the process of lipid peroxidation. Since the enzymes responsible for the activation step are expressed in the hepatocytes located around the central veins, these are the primary targets of carbon tetrachloride toxicity. Because of this mechanism, a single high dose

of carbon tetrachloride will induce massive necrotic cells death in the perivenous hepatocyte population in exposed experimental animals and humans. Interestingly, however, this tissue damage is repaired within only several days by regenerative proliferation of the remaining hepatocytes completely replacing their eliminated cousins and reconstituting the liver function without any remaining deficiency (*Figure 2.1A*). The damage within the hepatocyte population is sensed by a second cell population in liver, the stellate or Ito cells, which become transiently activated but return to a quiescent state after the liver regeneration process is completed. In summary, a single acute intoxication with carbon tetrachloride causes massive necrotic cells death in liver which is, however, completely repaired within a very short period of time by tissue regeneration without leaving any remaining damage to the liver function (*Hoehme et al., 2010*).

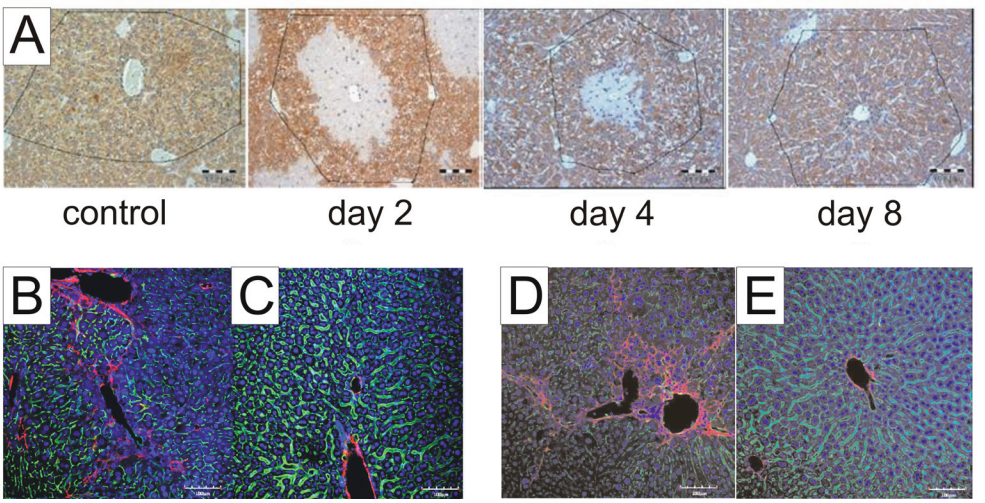

Figure 2.1 *Effect of CCl_4 in mouse liver. A: Effects observed in liver at the indicated days after a single high dose of CCl_4 (1.6 g/kg b.w., i.p.). Note the initial necrotic death of centrilobular hepatocytes followed by complete regeneration of the liver tissue by day 8. B, D: Effects observed after repeated application (6 weeks of CCl_4 administration; 3 weekly i.p. injections of 0.25 mg/kg CCl_4). B: Immunostain: red, smooth muscle actin which visualises activated stellate cells (myofibroblasts); green, sinusoidal endothelial cells and bile canaliculi; blue, nuclear DAPI stain. D: Immunostain: red: collagen type III, visualizing fibrosis; green and blue as in B. C, E: respective immunostains of control livers (Courtesy of Jan Hengstler; for further details see Höhme et al., 2010).*

A quite different situation is seen upon repeated doses given to the experimental animals of now smaller doses of carbon tetrachloride. In principle, the same cell killing effect occurs within

the primary target cell population but now only affecting a very small number of hepatocytes, namely those having the highest activity of the toxifying enzymes. In a process which is only partly understood, however, the hepatic stellate cell population now becomes permanently activated and is starting to proliferate. Simultaneously, in a feed-back mechanism, they release further stimulating cytokines and transform into myofibroblasts, which are most probably the cells that extensively produce the extracellular matrix material (e.g. collagen type III) that ultimately causes liver fibrosis, a serious complication, which is not seen after single acute treatment with the chemical. In synopsis, the primary damage to the perivenous hepatocytes population triggers a sequence of events, mediated by complex cell-cell interactions, that ultimately results in an irreversible manifestation of damage to the target organ, the liver (*Figure 2.1B-E*).

In principle, repeated dose systemic toxicity as opposed to acute toxicity may result from the pharmacokinetic behaviour of a chemical (its fate in the body) or from pharmacodynamic peculiarities (its effects in the body) not occurring after single exposure. The easiest explanation for repeated dose systemic toxicity not seen after single treatment is accumulation of the chemical in question, either because its only very slowly metabolised and subsequently excreted from the body or because it is trapped in certain compartments of the body, due to its physico-chemical properties. TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin), also known as the “Seveso poison”, is an example for a compound that is extremely stable in the body with a half-life between 7 and 15 years. We are chronically exposed to this agent through our diet and accumulate the highly lipophilic chemical in our fat tissue until equilibrium between uptake and excretion is reached after decades of exposure. Fortunately, the present exposure levels are so low that even under the given chronic exposure scenario adverse effects of TCDD are very unlikely and definitely not observable (*Schwarz and Appel, 2005*). A pharmacokinetic behaviour like that of the unwanted environmental compound TCDD is avoided in the world of pharmaceuticals and cosmetic ingredients. There are no drugs and other chemicals used in pharmaceutical products that are difficult to be metabolised and tend to accumulate in the body. Only under certain rare conditions so called ”idiosyncratic” toxic responses are seen for reasons that are only partly understood which may be at least in part - related to unexpected and uncommon pharmacokinetic behaviour of a drug. Idiosyncratic liver toxicity – even though very relevant in the clinic – is not investigated in **SEURAT-1** but may become a topic in a future phase of the SEURAT Research Initiative. Accumulation of a drug within a target organ, causing toxicity when a certain effect concentration is reached is not the only relevant effect related to pharmacokinetics. Defence mechanisms may be triggered after repeated exposures caused by phenomena like enzyme induction, epigenetic changes or adaptation at the molecular level. As a consequence the pharmacokinetic behaviour of a drug may change upon repeated exposure which may be associated with a change in its toxicity.

Similarly, the pharmacodynamic behaviour of an agent may change when given repeatedly, for various und often unpredictable reasons. In principle, two different mechanisms may be relevant: accumulation of damage within a single target cell or induction of a sequence of events

involving multiple cell types and/or organs that ultimately result in a delayed form of toxicity.

In the first case, adaptive response mechanisms triggered in the target cells after a single acute exposure to a toxicant may become overloaded after repeated exposures leading to delayed toxicity. This effect may occur, for example, if a protective molecule that is burned up as a result of the first toxic insult is too slowly re-synthesised to guaranty the required protective level at the following exposure(s). Numerous other mechanisms may also exist but, interestingly, only little solid knowledge on this seems to exist. The “omics” investigations planned in different projects of the **SEURAT-1** Research Initiative are potentially suited to shed some light on this problem, when they will study changes in gene expression patterns as a function of time after exposure and group the affected genes/proteins into functional clusters that are sequentially affected.

An example for the second mechanism, where a primary toxic effect triggers a sequence of other toxic responses, was given above for carbon tetrachloride-mediated induction of fibrosis in liver. Liver fibrosis is a serious health problem causing, amongst others, portal hypertension, and fibrosis can also affect other organs such as heart or lung. As described above, complex interactions between cells of different lineages is responsible for the induction of fibrosis upon repeated doses of the hepato-cytotoxicant. In this case, all cell types involved are present in the liver and fibrosis is induced intrahepatically. There are, however, examples, that more than one organ is involved in the genesis of a toxic response in the body: one is the carcinogenic aromatic amine 2-naphtylamine: this compound is acutely toxic because of its potential to transform haemoglobin to methaemoglobin, which has lost its oxygen-transport function. The compound is N-glucuronidated in liver, which eliminates its ability to form methaemoglobin. The glucuronate, however, is chemically unstable in urine in the bladder, where it accumulates prior to excretion, and decomposes to a very reactive electrophilic ionic intermediate which covalently binds to DNA of the bladder epithelial cells and induces mutations and cancer in this secondary target organ.

The few examples already demonstrate the complexity of reactions that may underlie toxic effects seen within organs in the body upon single or repeated exposure to a toxic agent. Successful replacement of animal testing for repeated dose toxicity is not just dependent on understanding biological processes, but also on how they are integrated and what the concentrations are at which adverse effects happen. While we often dwell on the flaws of animal models they do serve as integrators of multiple changes within the organism, integration that is often directly linkable to a human disease state. The **SEURAT-1** Research Initiative is aimed at collating our existing knowledge of biological systems, mechanisms of toxicity and interactions among systems to try make sense of non-animal data as predictors of risk for human toxicities. This should extend towards a greater understanding of human health. It will be a stony way to develop test systems that are able to improve our present testing strategies but it’s worth doing.

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2.5 Current State of the Art in Repeated Dose Systemic Toxicity Testing

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2.5.1 Repeated Dose Toxicity Testing as Part of the Risk Assessment Process

Risk assessment is a prerequisite to assure human safety. This process consists of three important steps: hazard assessment (consisting of hazard identification and dose–response assessment), exposure assessment and risk characterisation. In terms of cosmetic ingredients, the main goal of risk assessment is to answer the question whether the use of a chemical at a certain amount/concentration in a product represents an (un)acceptable safety risk to consumers. Crucial steps in the risk assessment process are the exposure assessment and the identification of a dose-response relationship.

With regard to exposure assessment, it is important to determine how much product and therefore ingredient is used, where it is used and the frequency and duration of use. The potential routes of exposure (dermal, inhalation, oral) need to be determined and where there is potential for absorption an understanding of systemic exposure (absorption and pharmacokinetic studies) may be necessary.

The risk that the exposure will cause adverse effects in consumers needs to be characterised, both for systemic and local toxicity endpoints. These endpoints range from acute, single exposure toxicity through to chronic, long-term exposure effects, genotoxicity and carcinogenic effects, teratogenicity and effects on reproduction and fertility. Local actions such as eye irritation and sensitisation are also important in the safety evaluation but are not the primary focus of repeated dose toxicity studies hence are not discussed further.

The so called hazard identification is the determination of the intrinsic toxic potential of chemicals and may be obtained from existing toxicological, clinical or epidemiological data on the substance itself (if the ingredient has a history of use) or related substances, e.g. the transfer (or read-across) of the hazard profile of one substance to another with similar structure.

It is also important to understand the relationship between the dose or concentration and the incidence or severity of response, i.e. dose-response assessment, which is used to establish the basis for predicting effects at various exposure levels. Key parameters of the dose-response assessment are, the No Observed Adverse Effect Level (NOAEL), threshold levels, shape of the dose-response curve and reversibility of effects.

When there is limited data to inform on the toxicity potential of a substance, tools such as

the Threshold of Toxicological Concern (TTC) and Structure Activity Relationships (SAR) can be helpful. TTC has been introduced in toxicology to indicate the threshold below which the chemical has no harmful effects. It relies on the concept that chemical structure defines the toxicity potential and groups substances based on structural features into potency categories. Existing data on various chemical classes of substances is then used to predict the toxicological potential of substances of undetermined toxicity. Although gaining acceptance in other areas, in the field of cosmetics, the TTC is not yet accepted by the Scientific Committee on Consumer Safety (SCCS) for safety assessment of cosmetic ingredients. SAR can also help establish a toxicity profile, either in a qualitative or quantitative manner by correlating the chemical structure and physical-chemical properties of a substance to similar compounds (analogues) with valid measured toxicity data. Although SAR has been used to fill data gaps such as for High Production Volume Chemicals, Food Additives and Fragrance Materials, there is little guidance provided on how to decide whether an analogue is appropriate for filling a data gap and even less is provided on how to read across data in a quantitative manner.

In case no sufficient existing data are available to inform on the hazard characterization and dose-response assessment, the lack of suitable alternative methods (see below) means that animal tests are currently required for providing the information. The information related to the process of testing and evaluating the safety of cosmetic ingredients is described in the Guidance on the safety assessment of cosmetics, updated by the SCCS (*SCCS 2010*). These studies are intended to provide a point of departure for the risk characterization step. The point of departure most commonly used for systemic toxicity safety assessments is a NOAEL, which is usually expressed in mg/kg body weight/day. This NOAEL is then compared to human exposure and a determination is made as to whether the exposure difference is sufficiently large, taking account of uncertainties in the safety data, so as to support the human exposure. For safety assessment of cosmetic ingredients it is generally considered that human exposures should be at least 100-fold below the animal NOAEL, taking account of inter- and intra-species variations in both toxicokinetic and toxicodynamic factors. Default uncertainty factors of 10-fold each applied in this regard may be refined in the case of knowledge of chemical specific data. On the other hand, additional uncertainty factors may be considered appropriate. The exposure difference (NOAEL versus human exposure) is described by the SCCS as the Margin of Safety (MoS) and as stated previously is the approach most commonly used for systemic toxicity safety assessment of cosmetic ingredients. Other methods and terminologies for risk characterisation include (but are not limited to) Benchmark Dose Analysis, Virtual Safe Dose, Tolerable Daily Intake, Margin of Exposure (*Filipsson et al., 2003; Rietjens et al., 2006*). Their use to characterise risk depends on the endpoint of concern and available data.

The repeated dose toxicity *in vivo* tests characterise the toxicological profiles of chemicals after daily exposure to graduated doses to several groups of experimental animals, one dose level per group for a pre-defined time span (e.g. 28, 90 days, 12, 18, 24 months). These tests provide information on possible adverse effects on organs, on dose-response relationships,

and on the reversibility of observed adverse effects. Animal tests provide information on a wide range of endpoints, as changes of many organs and tissues are taken into account (*ECHA, 2008a*).

Long-term repeated exposure to a chemical may result in persistent or progressively deteriorating dysfunction of cells, organs or multiple organ systems. The onset and progression of this toxicity is influenced by the responsiveness of the cell type (e.g. different regeneration capabilities of cells, such as hepatic and neuronal cells), interplay between different cell types, tissues and organs, including the concomitant contribution of toxicokinetics, hormonal effects, autonomic nervous system, immuno- and other complex systems.

The current animal tests for assessing repeated dose toxicity are described in the OECD (Organisation for Economic Co-operation and Development) guidelines for the testing of chemicals (*OECD, 2011*). They include: repeated dose 28 days oral, dermal and inhalation studies in rodents, 90 days oral, dermal, and inhalation toxicity study in rodents, 90 days and 1-year oral toxicity study in non rodents, and chronic toxicity study in rodents. Most of the available safety data on chemicals are currently based on these *in vivo* tests. For pharmaceuticals, pesticides and biocides the chronic toxicity studies are compulsory before the marketing of new products (it can be frequently found the 28-day studies are waived). For cosmetic chemicals, the most common repeated dose toxicity studies are the 28-day and the 90-day oral toxicity tests in rodents (*SCCS, 2010*).

The repeated dose toxicity tests may also provide information on e.g. the carcinogenic potential of a chemical, reproductive and/or developmental toxicity and toxicokinetics. There are specific tests, also repeated dose in their nature, for assessing these endpoints. However, these endpoints are not in scope for the present Research Initiative.

In general, *in vivo* studies concerning repeated dose toxicity appear to be effective in safeguarding public health. However, there are a number of limitations associated with these *in vivo* studies. The differences in metabolism and kinetics as well as in physiology and anatomy between different species may affect the outcome of the studies. Genetic polymorphism may not be covered as the group sizes are relatively restricted, and the polymorphisms may differ between species. However, it is worth mentioning that these issues can be addressed by using *in vitro* methods (*Adler et al., 2011*). Furthermore, the effects of age or possible disease-related parameters on toxicity are not easily covered in *in vivo* studies. In addition, the mechanisms of toxicity are not easily identified, and due to statistical and other problems intrinsic to *in vivo* toxicity testing, the doses used are generally higher than those humans are exposed to.

Only few surveys have explored the concordance of repeated dose toxicity between humans and animals. These studies have shown that e.g. for pharmaceutical compounds, the concordance of toxicity is higher when both rodents and non-rodents are used compared to studies comprised of only rodents (*Olson et al., 2000*), and many liver idiosyncratic effects

observed in humans are not recorded in rodents or non-rodent species (*Spanhaak et al., 2008*).

Nevertheless, as there are similarities between humans and animals, *in vivo* testing has proven to be relatively reliable and effective. In addition, these studies allow evaluating an integrated response in which all organs and toxicity endpoints are covered, including compensatory responses and its quantitative aspects. Therefore, its replacement imposes a great challenge.

2.5.2 Kinetic Considerations

A relevant factor when assessing the toxicity potential of a compound is its kinetics: absorption, distribution, metabolism and excretion (ADME), which affect the concentration encountered at the site of action. The knowledge of the toxicokinetic behaviour of a substance is a key piece of information, in particular when a new framework based only on non-animal alternative methods/strategies is sought. In the context of the Cosmetics Directive 2013 marketing ban deadline for repeated dose toxicity (*Anonymous 2003*), the European Commission established in 2010 five working groups tasked to evaluate the current status of development of alternative methods and future prospects in the five toxicological areas of concern (repeated dose toxicity, skin sensitisation, carcinogenicity, reproductive toxicity and toxicokinetics). The experts involved in the toxicokinetics working group concluded that, given this new framework, the information on toxicokinetics is essential for developing and designing more efficient testing strategies based on the knowledge of a compound's bioavailability by one of the relevant uptake routes, for performing *in vitro – in vivo* extrapolations, and for identifying clearance rates and the role of metabolites. In addition, *in vitro* biokinetic data recorded during an *in vitro* experiment will be crucial to derive the actual *in vitro* concentration. The uncertainty about the actual level of exposure of cells *in vitro* already present after a single exposure (due to the physico-chemical properties of the substance, its interaction with medium, cell and plastic devices) is enhanced after repeated treatments for prolonged times due to altered bioavailability or to physiological cellular processes induced by the treatment itself.

Physiologically-based biokinetic (PBBK) modelling, that takes into account physiological processes and compound-specific parameters, could ideally provide a full kinetics description of a compound, indicating the entire path from absorption to excretion, including possible metabolites, in any exposure scenario (*Andersen, 2003; Prieto et al., 2006, Blaauboer, 2003, 2008*). PBBK models are suitable for integrating the data obtained from *in vitro* and *in silico* studies into a biologically meaningful context as well as for *in vivo* extrapolation. It is also a suitable method for deriving data on the true intracellular *in vitro* concentrations of compounds. However, it should not be ignored that more data on *in vitro* biokinetics should be collected in order to build suitable models. The PBBK or other model cannot provide the answer without good data (*Adler et al., 2011*).

Currently there are some *in vitro* and *in silico* models available for most of the steps and mechanisms that govern the toxicokinetics of chemicals, but thus far only *in vitro* dermal absorption has been validated. The lack of means for obtaining *in vitro* data on the absorption after exposure via inhalation as well as on excretion is the major gap in kinetics. When all the steps and pieces of the puzzle will be available, it would be necessary to find the adequate modelling approach to integrate all the relevant information (*Adler et al., 2011*).

Kinetic modelling could be used as a part of new non-animal based integrated testing strategies that will need to be developed for predicting repeated dose toxicity.

2.5.3 Current Status of Non-Animal Methods for Target Organ Toxicity Testing

In the last few years, European legislation has been calling for the use of alternative methods to animal testing.

The fact that cosmetic ingredients are chemicals means that the data required for their safety assessments in cosmetics may already be available as a result of compliance with the provisions of the EU REACH chemicals legislation (*Anonymous, 2006*), except for some defined product classes (e.g., new or revised colorants, preservatives and UV-filters) for which the submission of safety data to SCCS is a legal requirement before their use in cosmetics is permitted. In the REACH legislation, article 1 promotes alternative methods for safety testing, article 25 states that animal testing must be used as a last resort, and encourages the exploitation of useful alternative methods. In addition, article 13 states that information on hazards (regarding positive results) and risks may be generated by suitable alternative methods that have not yet been taken up as official regulatory test methods, upon the condition that such methods fulfil the requirements of Annex XI (e.g., ECVAM criteria for the entry of a test into the prevalidation process (*Curren et al., 1995*)). If such methods are moreover validated, both positive and negative results from these methods will be accepted. Thus, REACH also provides a strong impetus towards the development, acceptance and use of alternative methods that could reduce the number of animals used for toxicological testing.

Furthermore, the European Commission Directive 2010/63/EU on animal protection also promotes the use of alternative methods and states that “an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available” (*Anonymous, 2010*).

However, despite this strong regulatory pressure, validated alternative methods that have been developed and taken up in the European legislation can today only be used to detect local (e.g. skin corrosion, irritation, absorption, and phototoxicity) acute effects, skin sensitisation (although not fully animal-free) and specific endpoints for genotoxicity *(ECVAM, 2011)*.

The complexity of regulations and data requirements across sectors and countries are posing additional challenges for implementation of the 3Rs (i.e. to reduce, refine and replace animal

testing). Although the UN Globally Harmonised System (GHS) was developed to promote regulatory consistency and efficiency among countries and sectors, better international harmonisation is needed. From an industry point of view, to have different data requirements in different parts of the world, means to perform different tests resulting in more money to spend. To overcome this problem, often the higher level of testing is chosen resulting in unnecessary animal testing as well as increased costs.

One of the major limitations of *in vitro* models in assessing toxicity after repeated exposures to a toxic substance is the impossibility to fully mimic the *in vivo* situation with all the interactions between cells/organs and the kinetics resulting in possibly differing toxicity response. The lack of knowledge of the toxicity mechanisms is a substantial drawback and, therefore, imposes problems in regards to the usefulness of *in vitro* tests in predicting the *in vivo* situations. From *in vitro* studies, it is also difficult to derive values (such as NOAEL) and thresholds that are needed for risk assessment (*Pauwels & Rogiers, 2010; ECHA, 2008b*). Currently, we are lacking clear understanding of the relevance of some of the disturbances (i.e. biochemical changes, up-down regulation of genes) observed *in vitro* and their impact on human health. Are they real biomarkers or simply adaptive responses to the insult? (*Boekelheide & Campion, 2010*). Furthermore, to date, the extrapolation of *in vivo* dose-response from *in vitro* concentration is still a problem (*Adler et al., 2011*) despite some attempts made to tackle this issue (*Verwei et al., 2006*).

The majority of the *in vitro* models available are based on cell cultures. These, however, generally have only a very limited lifespan, unstable phenotypes, undergo dedifferentiation, or functional properties may either be lost (both primary cultures and cell lines) or uncharacteristic, especially in case of cell lines (*Kim & Rajagopalan, 2010*).

In silico tools, in particular (Q)SAR ((quantitative) structure-activity relationship) models, may also be useful under certain circumstances. Repeated dose toxicity is a highly complex area and, therefore, it imposes significant challenges in building a single predictive (Q)SAR model. However, (Q)SAR models could prove useful in predicting effects associated with specific mechanisms. There are indications that suitable models for chronic toxicity predictions could be developed. The current models, some of which have been developed to serve the needs of the pharmaceutical industry, are preferably used as a part of an integrated strategy rather than as the sole prediction model of toxicity.

As an example of (Q)SAR models currently available, Derek Nexus (formerly Derek for Windows) is able to identify structural alerts relating to organ level effects such as hepatotoxicity (*Marchant et al., 2009*). However, further research is needed to broaden the capacity of Derek Nexus to identify an increased number of organ level effects. A statistically based expert system, TOPKAT, is, at present, able to estimate numerical values, e.g. lowest observed adverse effect level (LOAEL), that are essential in terms of risk assessment. The current model is developed from over 40 structural descriptors of five chemical classes. A further model has been developed to predict the maximum recommended therapeutic dose, data generally derived from human clinical trials (*Maunz & Helma, 2008*).

The real challenge for the *in silico* modelling of repeated dose toxicity is developing mechanistically relevant and transparent models. This may mean a move away from attempts to model LOAEL and similar quantities to a more fundamental grouping, or category approach. The purpose here is to develop rational and robust groupings of compounds that are likely to have the same effect at the organ level (the effect being responsible for driving the NOAEL). The basis for this grouping can then be related to what has been termed an "adverse outcome pathway" (*Schultz, 2010*). This pathway links the chemistry of the so-called molecular initiating event i.e. the interaction between xenobiotic and organism through the events that lead to an adverse outcome e.g. an organ level toxicity. Within this grouping, the relative effect may be modulated by cellular effects. For example, *Sakuratani et al., (2008)* demonstrated that within a category formed for 14 substituted anilines, the results of a 28-day repeated dose toxicity test conducted in rats was strongly related to haemolysis. The results of the analysis demonstrated that it was possible to correlate the values obtained for substituted anilines from 28-day repeated dose toxicity tests with quantitatively determined molecular properties. The application of adverse outcome pathways is linked to, and potentially will be stimulated by, the development of the OECD (Q)SAR Toolbox (*Diderich, 2010*). This software is freely downloadable from www.qsartoolbox.org. In the future it will be necessary to further develop rational profilers to group chemicals that relate to this endpoint, along with databases of repeated dose data to populate the groupings. Although biased towards pharmaceutical compounds, it is worth noting that the eTox project also aims to develop *in silico* models for organ level effects (*eTox, 2011*).

The complexity of the biological processes involved in systemic toxicity means that the development of non-animal systemic toxicity risk assessments cannot rely on a direct replacement of the animal tests currently used. The availability and status of alternative methods for repeated dose toxicity has been recently reviewed in the context of the Cosmetics Directive 2013 marketing ban deadline as mentioned above. The *in vitro* methods in this area have been developed with the aim of producing stand-alone methods for predicting effects in specific target organs. Therefore, the report reviews the *in vitro* models available for the most common target organs and target systems for toxicity (liver, kidney, lung, central nervous system, cardiovascular system and immune system), although it is recognised that there are many more target organs for which unfortunately less or no *in vitro* methods are available. The experts concluded that none of the models described can currently be applied for quantitative risk assessment for repeated dose toxicity. In this regard, the report also addressed the challenges for the development of alternative approaches for quantitative risk assessment of cosmetic ingredients. In addition, since the overall aim is to predict toxic effects on humans, not solely to obtain similar data as animal-derived, the experts concluded that any new approach needs to be based on an increased understanding of the perturbations in cellular processes that lead to adverse effects and has to be based on human biology rather than attempting to emulate a rat (*Adler et al., 2011*).

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3 SEURAT: VISION, RESEARCH STRATEGY AND EXECUTION

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

"The goal of mode-of-action, human biology-based testing is not to generate batteries of tests to provide a prediction of animal toxicity test results for various endpoints. Instead, these methods are intended to determine regions of exposure that will not cause any adverse responses in exposed human populations."

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3.1 Introduction

The SEURAT initiative - Safety Evaluation Ultimately Replacing Animal Testing - was introduced in 2008 by the Health Directorate of the European Commission's Directorate General for Research and Innovation. The aim was to devise and implement a comprehensive EU research programme that will drive a major overhaul in the chemical safety assessment paradigm, ensuring the greatest protection of human health without having to experiment on animals. The initiative is expected to take many years, perhaps decades, and will require significant resources. However, rapid advances in life sciences and a strong desire among stakeholders to embrace change suggest that SEURAT is indeed feasible.

The first execution phase, **SEURAT-1**, was successfully launched in January 2011. It comprises a cluster of five complementary research projects, a data handling and servicing project and a coordination action and is co-financed by the FP7 Health Programme and the European Cosmetics Association (*Colipa*), through a new model of public-private partnership. Over the next 5 years, over 70 research institutions will work together towards the replacement of repeated dose systemic toxicity testing on animals. The ultimate aim of this first cluster is to deliver a proof-of-concept to show how the latest scientific tools and knowhow can be combined to deliver decision support systems for safety assessment.

In this chapter we endeavour to further elaborate SEURAT, by proposing a *Vision* - describing what a future safety assessment paradigm should comprise, a *Strategy* - defining the underpinning scientific concepts and approach, and an *Execution* plan - that outlines the main elements of the research programme to be undertaken. In this context we also describe the research priorities of the first execution phase, **SEURAT-1**, and propose how the results and momentum can be carried forward to the next phase, **SEURAT-2**.

3.2 The Vision

The SEURAT vision is to fundamentally change the way we assess the safety of chemicals, by superseding traditional animal experiments with a predictive toxicology that is based on a comprehensive understanding of how chemicals can cause adverse effects in humans.

The vision foresees safety assessment frameworks that optimally combine a range of reliable and robust experimental (*in vitro*) and computational tools in a purposeful manner to deliver the relevant information needed for decision making. These predictive toxicology tools will associate substances of concern with a new taxonomy of toxicological hazard categories, and they will predict the likelihood of any adverse health effects as a function of exposure, for different sub-populations. The uncertainty of these predictions will be sufficiently characterised as to facilitate effective risk management and communication, with the appropriate degree of

precaution. The predictive tools will be widely available, affordable, and reliable so that every substance destined for commerce will be sufficiently evaluated in good time, at a reasonable cost, and in a consistent manner. To facilitate trade and the global market, safety assessment frameworks will be established and harmonised at international level, allowing them to be implemented in all jurisdictions. The knowledge gained from safety assessment of new substances will be fed back into the product development process, thereby improving human safety evaluation, driving innovation, increasing consumer choice, promoting sustainability, and improving industrial competitiveness.

3.3 The Strategy

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 mode-of-action framework (*Boobis et al., 2008*) is based on the premise that any adverse human health effect caused by exposure to an exogenous substance can be described by a series of causally linked biochemical or biological key 'events' that result in a pathological endpoint or disease outcome. An 'adverse-outcome-pathway' is a very similar concept proposed by the computational toxicology community (*Ankley et al., 2010*), where the linking of a chemical with a pathway that leads to an adverse human health or ecological outcome is determined by its ability to trigger the associated 'molecular initiating event'. Another related framework is that of 'toxicity pathways' introduced by the NRC (*Krewski et al., 2010*), where in this case the description of toxicological processes tends to focus on early events at the molecular and cellular level. Thus one can consider toxicological pathways as critical upstream elements of a more expansive mode-of-action or adverse-outcome-pathway description of how a chemical can compromise human health *(Figure 3.1).*

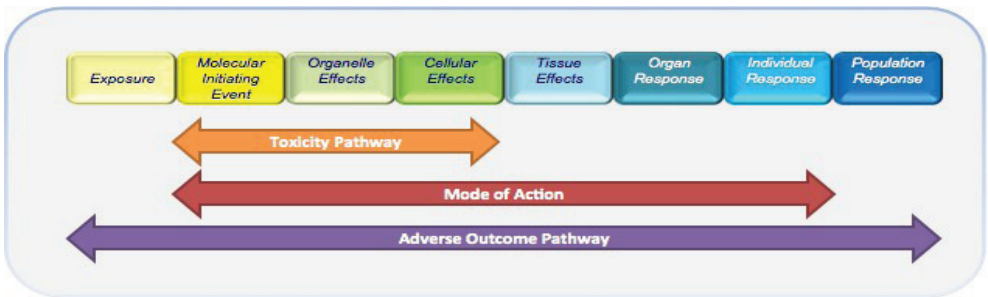

Figure 3.1 *Schematic illustration of a sequence of events contributing to an Adverse Outcome Pathway, including the Mode of Action and Toxicity Pathway as sub-sequences.*

Mode-of-action theory is still emerging but there are already a number of important principles that have shaped the SEURAT research strategy. The first is that every toxicant can be associated with one or more mode-of-action categories. To facilitate this, however, a suitable ontology that describes all the possible modes of toxicological action needs to be developed by harvesting and organising the wealth of knowledge and information available from the literature on well studied chemicals and pharmaceuticals. Systematically checking 'reference' chemicals against mode-of-action categories will help challenge and refine the mode-of-action ontology as it emerges, and will identify a wide range of key biological events and pathways that should be represented in relevant experimental (*in vitro*) and computational models.

The framework assumes that many modes-of-action share common key biomolecular or biological events. Thus it is the particular chain of causally-linked events that makes a mode-of-action unique. In the case where a substance is promiscuous and could trigger multiple modes-of-action, the concentration and persistence of the substance at the initiation sites will dictate the modes-of-action that will tend to dominate. Thus, for example, chronic low-dose effects are likely to be quite different from high-dose acute effects. Special consideration needs be given therefore to characterising dose-response relationships, to describe how and when mode-of-action transitioning may occur for a single substance, depending on factors such as exposure dynamics, site of action, genetic and epigenetic predisposition or inherent phenotypic vulnerabilities.

Another principle that must be considered concerning mode-of-action theory is that many key events and pathways are common to many cell types throughout the human body. Thus although the same substance can cause different pathological outcomes in different tissues, the upstream event, such as mitochondrial inhibition or generation of reactive oxygen species, may be common to the modes-of-action triggered at each site. On the other hand, certain modes-of-action involve key events or pathways which are associated with specific biological functions expressed by particular cell types. The presence of metabolising enzymes in liver cells which may bioactivate exogenous chemicals to produce toxic metabolites, or the presence of cell membrane transporters required for the uptake of certain toxicants are examples illustrating cell-type specific toxicity. Similarly, the presence of receptors for neurotransmitters in neuronal cells which can be targeted by toxicants is another example of cell specific properties that can be implicated in a toxicological mode-of-action.

Although many toxicological modes-of-action are conserved across mammalian species, there will likely be many situations where for example, rodent or tumour derived cell lines will fail to capture essential aspects of human biology. Attention needs to be given therefore to the development of experimental models based on properly conditioned human primary cells or differentiated stem cells. In addition, modelling a toxicological mode-of-action in a holistic fashion will require the emulation of downstream events that manifest themselves as pathology at the tissue level. Simple cell-based *in vitro* models will not be sufficient

Table 3.1. Key elements of SEURAT-1
Selection of well studied chemicals with evidence of chronic systemic toxicity. Hypothesis driven approach to elucidating modes-of-action and identifying associated key events and biomarkers.
Emphasis on *in vitro* models that capture important modes-of-action directly relevant to human physiology. Exploit stem cell technology to develop *in vitro* systems with cellular diversity to model higher level functions.
Development of fit-for-purpose *in vitro* assays suitable for High Throughput Screening (HTS) implementation. Use of bioreactors to engineer tissue comprising multiple cell types to model complex toxicological processes.
Biokinetic modelling to extrapolate between *in vitro* test concentrations and repeated dose organ exposure *in vivo*. Computational toxicology to associate chemicals with molecular initiating events and describe metabolism
Use of high content analysis tools including 'omics to describe modes-of-action at the molecular level. Systems biology approaches to model mode-of-action dynamics at the molecular scale for quantitative analysis.
Proof-of-concept exercise to demonstrate a mode-of-action based integrated test system to predict sub-chronic liver toxicity Feasibility study to show how test data can be used in a safety assessment context

for this purpose and thus 3D tissue models will be needed to reproduce the more apical biological processes or endpoints. These 3D tissue models will be produced experimentally in bioreactor systems, or virtually using computational biology approaches. Such models will not only allow the qualitative association of a chemical with one or more modes-of-action, but will also serve to quantify dose-response relationships. Complementing the cell and tissue models, computational chemistry, quantitative structure-activity relationships (QSARs) and chemoinformatics tools will provide the means to understand and predict key biochemical events such as protein binding and metabolic transformation. However, these advanced experimental and computational approaches may be limited if they are overly reductionist or simplistic, thus failing to capture aspects such as hormonal regulation, tissue innervation, immune surveillance, blood circulation and metabolic turnover.

An important aspect of the SEURAT strategy will be the emphasis placed on understanding and predicting the *in vivo* biokinetics of exogenous chemicals. Quantifying the dose in different target tissue compartments as a function of time and exposure conditions, will be a fundamental requirement of any predictive toxicology paradigm. The expectation is that most chemicals are not likely to be harmful to only one specific cell or tissue type, but that in fact most apparent specificity-of-action can be explained by the bioavailability of the chemical at different anatomical sites, dictated by how it is absorbed, distributed, metabolised and excreted in the human body. Experimental and computational tools to profile chemicals, for example, in terms of their affinity to bind to proteins, their metabolic stability, and their ability to diffuse or be transported across biological barriers, will provide the necessary input for physiologically based biokinetic models that will ultimately predict chemical fate *in vivo*.

Establishing a comprehensive description of the mode-of-action domain is a challenging but necessary element of the strategy that will require the use of advanced discovery and modelling tools. Identifying the key biological events and biomarkers that comprise a particular mode-of-action, and elucidating the relationship between these events will benefit greatly from high content functional analysis tools such as transcriptomics, proteomics, and molecular imaging. High throughput screening can play an important role in generating reference data using more traditional assay formats, whereas microelectronic and optical biosensing technology will be necessary to monitor the dynamic response of biological models in a non-invasive manner. Aiming at a more quantitative description of a mode-of-action and in particular, defining the array of conditions that must be met to progress towards an adverse outcome, or that might result in system recovery, will require mathematical models of sufficient complexity. Systems biology theory and tools will provide a strong basis for these models that will need to take different phenomenological aspects into account, such as the stochastic nature of many biological systems.

As the mode-of-action framework becomes more established, and the range of validated models grows, an increasing number of chemicals can be profiled to establish to which mode-of-action categories they belong. This will then facilitate read-across within categories and provide the basis for ultimately predicting hazard threshold values, akin to *in vivo* no-effect levels. Initially, assessment frameworks exploiting such predictions are likely to apply quite conservative uncertainty factors. However, as prediction algorithms are improved and validated, and the description and quantification of the uncertainty is more thoroughly addressed, it is likely that factors can be more optimally defined. Feasibility (proof-of-concept) studies will help pull together the various components of a testing strategy in a purpose-driven fashion, and will be an important instrument for engaging the regulatory community and promoting uptake of SEURAT approaches.

3.4 The Execution

3.4.1 The First Step, SEURAT-1

The first execution phase, entitled SEURAT-1, has a broad and highly ambitious work programme that aims to prove the scientific and technological concepts underpinning the SEURAT strategy. The key elements of the **SEURAT-1** Research Initiative are summarised in *Table 3.1*, and are being addressed by the cluster of six individual research projects described in Chapter 4. The overall emphasis is on the identification and elucidation of modes-of-action related to repeated dose systemic toxicity in humans, and the development of experimental and computational models that effectively capture the related pathways and key biological events. A set of reference chemicals is being compiled, which have been thoroughly investigated regarding their chronic toxicological action in animals, and in humans if possible, and this information will be used to propose an initial mode-of-action framework to which the various research activities can refer. The chemicals will also be supplied throughout the cluster as controls for *in vitro* model characterisation and assay development.

Significant effort will be invested in basic research concerning both embryonic and induced pluripotent stem cells, of human origin, with the intention of devising optimal maintenance and differentiation protocols that deliver large quantities of well characterised, stable, and reproducible cell lineages, which express the important phenotypic properties and functions found *in vivo*. An important goal of this work is the production of a comprehensive set of genetically engineered stem cell derived models which express light-producing enzymes or proteins on triggering of certain signalling or metabolic pathways *(Figure 3.2)*. Bioreactor technology will be employed to engineer 3D tissue constructs *in vitro* in an attempt to capture the intricate interactions between different cell types present in an organ that must work in unison to maintain homeostasis and function. It is anticipated that such systems will be required to represent more complex modes-of-action and to move towards more predictive systems from which chemical activity/effect levels can be derived.

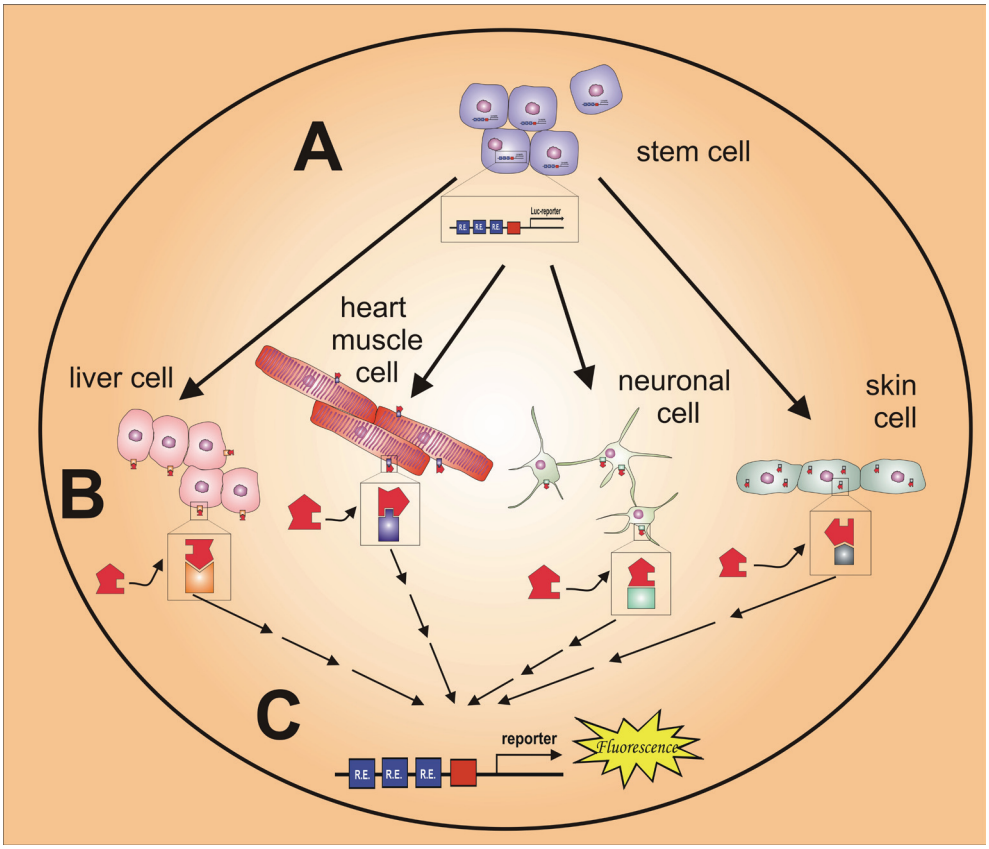

Figure 3.2: *The use of genetically engineered stem cells derived in vitro models for toxicity screening A: Cell lines are generated from human stem cells carrying reporters for relevant signalling or metabolic pathways associated with different modes-of-action B: Reporter cells are differentiated into cells of different lineages related to different target tissues of the liver, heart, muscle, central nervous systems, skin, etc. C: Differentiated reporter cells are incubated with test chemicals (red blocks) which may interact with different "receptors" (coloured blocks) thus triggering reporter activity that is monitored by measuring luminescent light.*

Transcriptomics and proteomics (*in vitro*) are being employed to dig deeper into the underlying molecular processes associated with selected modes-of-action, and this data and information will be used to guide the definition of systems biology models which capture the process dynamics and allow quantitative analysis and prediction of adverse pathway perturbation. As the mode-of-action framework is refined, and more key biological events are identified, new biomarkers of effect will be investigated that can be incorporated into assay systems. In addition, the intention is to exploit novel biosensing and imaging techniques to more effectively detect biomarkers, thus improving on more traditional read-out approaches.

Apart from the systems biology modelling, computational toxicology methods will also be applied in two specific areas, namely, biokinetic profiling and structure-activity relationships. The biokinetic profiling will centre on the use of Physiologically Based BioKinetic (PBBK) approaches to model both the *in vivo* and *in vitro* fate of exogenous chemicals, in terms of their adsorption, distribution, metabolism and excretion. This will allow *in vivo* to *in vitro* exposure extrapolation for a limited set of chemicals for which the relevant intrinsic/extrinsic properties are known, or can be determined (e.g. lipid-water partitioning coefficient, protein binding affinity, metabolic clearance rate). Regarding Structure-Activity Relationships (SARs), the attention here will focus on finding associations between the structural features of a chemical and its ability to trigger the key biomolecular events that initiative toxicological responses that may lead to adverse health outcomes. Forming chemical categories based on combined structure-activity descriptors will ultimately facilitate more rapid and robust hazard profiling of chemicals and read-across between chemicals which have a similar mode-of-action.

The **SEURAT-1** Research Initiative will deliver many important computational and experimental tools, and related knowhow, that will be critical components in predictive toxicology approaches. To demonstrate the potential of these tools and how they can be assembled in an integrated manner, the cluster will undertake a proof-of-concept exercise to demonstrate how a mode-of-action based testing strategy can be used to predict aspects of repeated dose target organ toxicity. In addition, a feasibility study will also be carried out to show how test data derived from such systems can be used in a safety assessment context. In doing so, the intention is to engage regulatory scientists and stakeholders in a practical dialogue aimed at building confidence in the tools, identifying important sources of uncertainty, and deciding on how to best progress the field to foster uptake and acceptance of the new methodology.

3.4.2 Next Steps, SEURAT-2 and Beyond

Successful completion of **SEURAT-1** will lay the foundation for follow-on efforts in **SEURAT-2** that will broaden the toxicological, chemical, and regulatory domains addressed, as illustrated in Table 2. The mode-of-action framework will have been well established, but will be limited in scope, covering mainly repeat-dose toxicity associated with primary organs. Thus the mode-of-action ontology will need to be further expanded by harvesting existing knowledge, and generating new knowledge where gaps exist, to cover other adverse health effects linked for example to cancer and reproduction.

Exploration of this broader toxicological domain will need a more extensive range of cell models and engineered tissues that capture important biological processes and function, that can be used not only to investigate and confirm modes-of-action, but which could also be used as a component of an integrated test system. Genetically engineered stem cell models that can be used for event-specific gene-reporter assays will facilitate this greatly, for example. **SEURAT-2** will have to consider a larger number of chemicals taken from a wider chemical space, in order to cover more diverse physicochemical properties, modes-of-action, and

Table 3.2. Keys elements of SEURAT-2
Broaden the toxicological mode-of-action ontology to cover other adverse effects Address the issue of population diversity regarding predisposition and susceptibility
Expand the inventory of cell models to cover other tissues and physiological processes Develop stably transfected stem cell models for reporter gene assays sensitive to key pathways
Cover a greater diversity of chemical type, structure and classes Broaden the chemical domain to cover an extensive range of industrial sectors
Scale up testing using High Throughput Screening (HTS) and High Content Screening (HCS) to generate more research data on a large set of reference chemicals Implement standalone computational workflows for virtual screening/profiling
Refine biokinetic and systems biology models to give more accurate and comprehensive predictions for a larger chemical space Assemble tools to realise integrated toxicological hazard prediction systems for a wide range of regulatory endpoints
Undertake a comprehensive evaluation and demonstration programme based on typical safety/risk assessment scenarios

related health effects. By tackling a wider chemical space, **SEURAT-2** will also be relevant for a number of different industrial sectors and legislative areas.

Broadening the toxicological, chemical, and regulatory domains to be addressed in **SEURAT-2** will require the generation of high quality *in vitro* datasets on large numbers of reference chemicals. Therefore experimental activities will need to be scaled up through the exploitation of High Throughput and High Content Screening (HTS/HCS) platforms, including *in situ* biosensing, imaging and '-omics'. Moreover, computational tools will require further development, refinement and integration to broaden their applicability domain and improve their predictive power. On completion of **SEURAT-1** it is likely that biokinetic modelling of Adsorption, Distribution, Metabolism and Elimination (ADME) of exogenous chemicals will not yet be sufficiently developed and thus **SEURAT-2** will need to invest further in this area if overall progress is not to be hindered. In addition, systems biology modelling will also have to be further improved and expanded in order to effectively link processes at the molecular, cellular, tissue, organ and organism levels, in order to make accurate quantitative predictions of *in vivo* effects from, for example, *in vitro* data. All this will need to be supported by the definition and implementation of computational workflows that formalise processing steps and decision making logic for a more consistent application of assessment methodology

in a context specific manner. Such workflows will ultimately drive the assessment process, commencing with virtual screening steps to use existing information and chemoinformatics to associate a chemical with specific mode-of-action based hazard categories, followed by targeted *in vitro* testing and computational analysis to elaborate dose-response relationships and to predict quantitative points of departure, such as no-effect levels. This should provide the basis to undertake a comprehensive programme of evaluation and demonstration, to consider a range of safety/risk assessment scenarios that can be effectively tackled with the new methodology. Moreover, key elements and methods might be subject to more systematic validation, if required to facilitate scientific and regulatory acceptance.

Uptake and application of SEURAT methodology for safety assessment will begin modestly on a proof-of-concept level within **SEURAT-1**, but will continually expand in both depth and scope throughout **SEURAT-2** and beyond. Possible application areas in the relatively near future include satisfying Classification, Labelling and Packaging (CLP) requirements, or supporting a weight-of-evidence analysis or read-across in a Chemical Safety Assessment under REACH. It is likely that novel tools and safety assessment frameworks deriving from SEURAT will be initially implemented and evaluated in parallel to more traditional approaches. This will identify any shortfalls, build confidence, and define good practice for better safety evaluation that will ultimately replace animal testing.

The transition to **SEURAT-2** represents the expansion and application of the concepts and tools proven in **SEURAT-1**. It will require a substantial scaling up of efforts to engage a wider section of the scientific community in a critical mass of complimentary collaborative-research projects. It is recommended that this be facilitated through the establishment of a dedicated research programme for innovative toxicity testing and safety assessment within the Common Strategic Framework for EU Research and Innovation. This should not only provide the necessary funding, but also the right instruments to support and coordinate large-scale strategic actions complimented by smaller targeted projects, and lever the resources and expertise of industry through public-private partnerships. In addition, it is imperative that the new EU research programme be positioned squarely within an international context, to join forces with complimentary initiatives in the USA, Canada, Japan and elsewhere, and to facilitate the work of international organisations such as the OECD and WHO.

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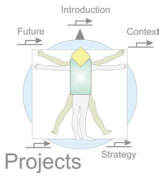

4 THE PROJECTS

"This unique research collaboration represents an important step in our on-going efforts to finding non-animal testing methods for cosmetic product ingredients. Together with the European Commission, we are enabling top scientists to come together from all around the world in order to make progress in this complex area of safety testing."

Colipa's Director General, taken from the Statement "Unique Research Initiative launched" published on 1 March 2011.

http://www.colipa.eu/news-a-events/statements.html (accessed 1 June 2011)

4.1 Introduction

This chapter provides a comprehensive overview about the projects of the **SEURAT-1** Research Initiative. 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 projects did start on 1 January 2011. Hence, as this first volume of the Annual Report

is issued early in the life of the cluster, it focuses on the plans, challenges and the results expected for the first year rather than progress made in each of the projects. Each project description is organised in the same way: (i) definition of the scientific objectives, (ii) state-of- the- art summarizing the available knowledge in the field, (iii) the approach how to reach the objectives, and (iv) the innovative aspects with respect to the scientific concept, used methodology, anticipated results in the long run, and possible applications. 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 summarizing the main activities in each of the projects as well as on the cluster level. This is followed by a description about the interactions between the different projects. The chapter is finished with an overview about the planned training activities (workshops and summer schools) and activities to promote the dissemination of knowledge.

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

Delphine Laustriat, Karine Giraud-Triboult, Sébastien Duprat, Marc Peschanski

4.2.1 Introduction

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

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

Transformed cell lines and primary cells sampled from donors are not optimal biological resources for high throughput testing of toxicity pathways

One main question raised by the acknowledgement that the “toxicity pathways” paradigm may allow shifting largely from animal-based to cell-based assays for industrial-scale testing of chemicals, lies in the quality and standardisation of biological resources on which such an endeavour can be most solidly based. Typical *in vitro* experiments make use of either transformed, immortalised cell lines or of primary cells that are isolated directly from tissues (*McNeish, 2004*). It is obvious that cells of human origin should be preferred for the sake of relevance, as far as they are accessible. Transformed cells, on the one hand, are accessible in unlimited amounts and usually easily maintained as they readily grow in standard culture conditions. However, although a large number of cell lines of tumour origin are available in commercial libraries, it is often not easy to access cells that exhibit specifically a phenotype and stage of differentiation of interest. Primary cells that have been immortalised –e.g. using hTERT- may be more reliable in that sense, but they often appear phenotypically unstable over passages required for toxicity screening. Relevance of transformed cells to the physiological conditions is also a major concern as they are usually genomically altered, often aneuploid, and extrapolation of results to their normal counterpart may not be straightforward. Even though they may exhibit general features of a cell identity of interest, they often do not ideally recapitulate the phenotypes and mechanisms that would be observed in their normal cell counterparts. Primary cells, on the other hand, offer a more relevant model system as they are supposed to express the exact phenotype of interest. This, however, should sometimes be qualified, as some of those cells – e.g. hepatocytes- tend to lose some phenotypic traits over time and passages in culture. Primary cells are genotypically normal and, if naturally expandable – which is directly dependent upon the cell type of interest – may be scalable up to some medium to high throughput. This is often not the case, though, which makes them then unfitted for the task considered here. A further significant limitation applying to primary cells is their phenotypic diversity arising from genetic variation between different donors. First, many phenotypes of interest are essentially impossible or at least very difficult to access. Second, sampled cells cannot be directly scaled at will. Third, and most importantly, donors are different from one sampling to another, leading to an uncharacterised genetic impact on the results and to quite obvious batch-to-batch variations in the overall quality of the cells. This analysis has led us to discard both cell types for the ***SCR&Tox*** programme and to rely uniquely on pluripotent stem cells that seem to us to exhibit optimal biological characteristics for toxicity pathways testing at the industrial scale (*Figure 4.1*).

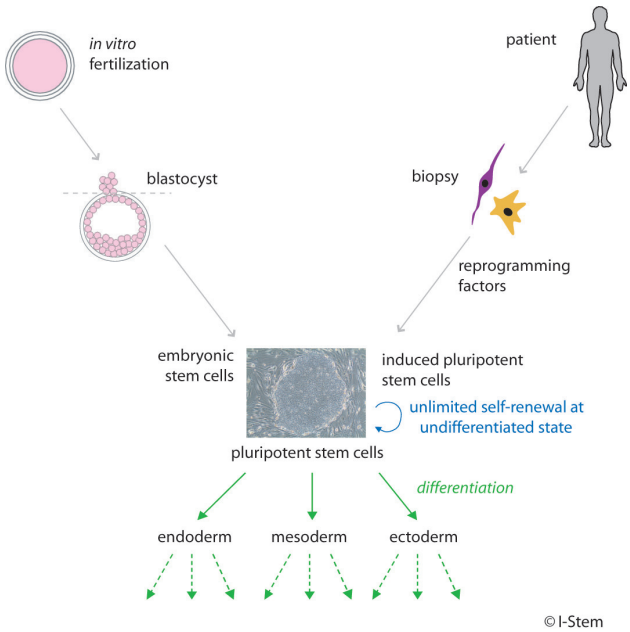

Figure 4.1 *Scheme for the derivation of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs).*

Pluripotent stem cells are the most accessible and relevant resource for cell-based assays of toxicity amenable to industrial scale

Pluripotent stem cells, whether of embryonic origin (ES cells) (*Thomson et al., 1998*) or induced to pluripotency by genetic re-programming of somatic cells from donors (iPS cells) (*Takahashi et al., 2007; Takahashi & Yamanaka, 2006*), share a number of attributes that, in our view, make them uniquely suitable for meeting the challenges of the new toxicity testing paradigm. These cells – of human origin– are either physiological (ES) or else apparently similar to physiological cells (iPS), thus providing some guarantee for relevance (*Hoffman & Carpenter, 2005; Yu and Thomson, 2008*). Because they are formally immortal, they can be obtained in any requested amount from any chosen donor. Repeatability of testing on a single genetic background is thus perfectly feasible. They can also be obtained in similar phenotypic conditions from any number of different donors, opening the path for studies of a potential inter-individual variability of responses. Pluripotent stem cells are, by definition, amenable to differentiation into almost any cell type, of any lineage, at any stage of differentiation, whenever one has identified a workable protocol for *in vitro* processing of the cells. It is, in particular, possible to obtain not only fully differentiated cells of any organ but also intermediate precursors. Those precursors have often proved quite interesting for long-term scalable analyses because they can be maintained for many passages (e.g. over 100 for human ES-derived neural precursors) without loss of lineage-specific traits and may, therefore, be instrumental for analysis of repeated-dose toxicity. Pluripotent stem cells can be used for parallel analysis of the effects of toxicants on cells representing different organs of interest, on a same genetic background. They are also discretely amenable to genetic engineering either at the undifferentiated stage

or as self-amplifiable intermediate precursors, allowing for provision of specific properties of interest such as gene constructs indicative of the action of chemicals or else transcription or signalling factors promoting desired phenotypic changes. The ***SCR&Tox*** programme is, therefore, entirely based upon human pluripotent stem cell lines. It will analyse in parallel human ES and iPS cells because of their complementary interest, the former being much better explored and understood already and having in particular demonstrated robustness and reliability at the industrial scale, the latter being potentially more versatile, in particular for large-scale analysis of the impact of human polymorphisms on responses to toxicants.

4.2.2 Objectives

The use of this "toxicity pathways" paradigm has major and diverse impacts on the way toxicity testing is to be performed and what it can provide. As reflected by its acronym -Relevance (and reliability), Efficiency (throughput), Extension (diversity and scalability), opening the path toward Normalization (validation) of a new system of toxicology testing-, the ***SCR&Tox*** programme focuses on this new paradigm and relies on the working hypothesis that pluripotent stem cells constitute a new generation of models of choice to accompany this tremendous shift in toxicology testing.

Fully integrated within the **SEURAT-1** Research Initiative, with the other consortia that will each bring expert bases for different aspects of the new strategy, the aim of the ***SCR&Tox*** programme is to PROVIDE biological and technological resources needed to assay toxicity pathways *in vitro* and to DEMONSTRATE on industrial platforms that these resources can be reliably and robustly implemented at the required scale (*Figure 4.2*).

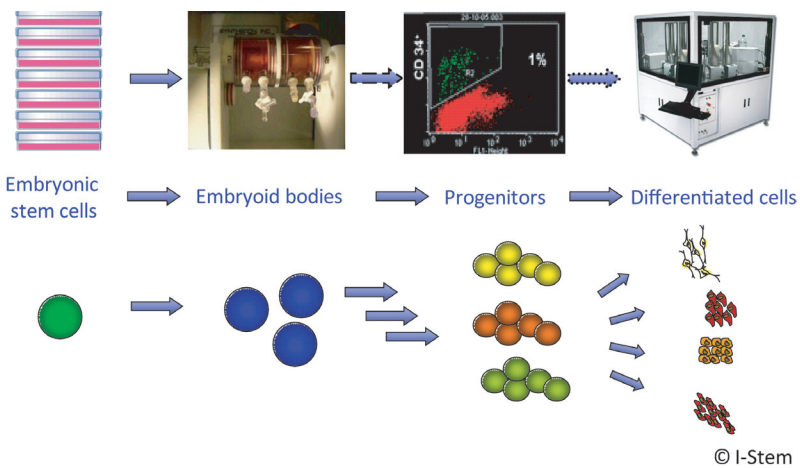

Figure 4.2 *Schematic representation of the* ***SCR&Tox*** *strategy for the mass production of biological resources to be used in toxicity testing.*

Our scientific objectives are:

- To obtain the pluripotent stem cell lines required for the programme both in terms of quality, i.e. ES and iPS from a sufficient number of donors, and quantity through implementation of scalable production technologies
- To design and implement optimal protocols for differentiation of pluripotent stem cells along 5 different lineages (liver, heart, CNS, epidermis and muscle), to fully terminally differentiated cells. For manufacturing and specific assay development reasons, some of the cell types, i.e. CNS, epidermis and muscle will be characterised in an additional stage, an intermediate precursor stage.
- To design and implement engineering and production methods to optimise those differentiated cells specifically for toxicity pathways assays
- To identify and optimise, or otherwise develop and standardise technologies for exploring cell functions relevant to toxicity pathways assays
- To implement on the bench cell-based assays of toxicity pathways identified by the other consortia of the **SEURAT-1** Research Initiative using optimised and newly developed technologies
- To promote biological resources to scale, reliability and robustness for implementation on industrial HTS platforms
- To develop at least one stem cell-based assay of a toxicity pathway validated on the bench for implementation on industrial HTS platforms
- To demonstrate the value of at least one prototype of a stem cell-based toxicity pathway assay on industrial HTS platforms
- To enter at least one prototype of a stem cell-based assay of a toxicity pathway into normalization and validation
- To address the potential phenotypic diversity of cell lines and select a robust panel of cells for large scale preparation of test cultures that are suitable for high throughput screening.

4.2.3 State of the Art

The ability of stem cells to meet the challenges of the paradigm shift in toxicology has attracted enormous attention in recent years. Although initiatives had been launched before in Europe and in the US, the most impressive demonstration of this new focus in toxicology has been the report published by the National Research Council of the US National Academies in 2007 (*Committee on Toxicity Testing and Assessment of Environmental Agents, 2007*): "*Toxicity*

Testing in the 21st Century, a Vision and a Strategy". This seminal document clearly stated that "*toxicity testing was approaching a pivot point*" as advancement of science and technology "*could transform (it) from a system based on whole-animal testing to one founded primarily on in vitro methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin.*" Based upon the acknowledgement of the existence of "*toxicity pathways*", i.e. "*cellular response pathways that, when sufficiently perturbed, are expected to result in adverse health effects*", this report called for the development of stem cell-based assays in high throughput formats. CIRM, The Californian Institute of Regenerative Medicine (*Workshop, 2008*), issued the following year its own report of a workshop dedicated to "*Stem Cells in Predictive Toxicology*", the main conclusions of which concurred with those of the NRC, underlining the enthusiasm of participants "*about the potential for stem cells to provide superior model systems for predicting toxicity*".

European reflections and efforts paralleled those from the US. The Stem Cells for Safer Medicine organization (*SC4SM*) in the United Kingdom was established in 2007, including three major pharmaceutical companies, in order to facilitate such cooperation, with the goal of developing superior toxicology models from human stem cell sources. Over the different projects funded by the EU FP6 and 7, 5 already related to stem cells and the development of toxicity tests (*ReProTect*, *CarcinoGenomics*, *VitroCellomics*, *InvitroHeart and ESNATS*) (*AXLR8, 2010*). Up to now, however, these initiatives ambitioned each no more than to provide a partial response to a discrete question (e.g. specific cell replacement in available assays).

The **SEURAT-1** Research Initiative expresses the ambition of an integrated response to the announced paradigm shift. Within that large (multi-line) framework the ***SCR&Tox*** programme proposes to make use of the two major attributes of pluripotent stem cells, namely unlimited self-renewal and pluripotency, to provide a reliable and reproducible source of human cells for *in vitro* assays useful for predicting toxicity of pharmaceutical compounds and cosmetic ingredients in the Human. It is our contention that baseline information available on embryonic stem cells and induced pluripotent stem cells allows our consortium to address all issues related with the biological and technological resources required to meet that goal.

Human pluripotent stem cell lines, ES and iPS, represent an unlimited biological resource for assays at an industrial-scale

Embryonic stem cell lines are established from the human blastocyst inner cell mass (*Heins et al., 2004; Thomson et al., 1998*). Under the right conditions ES cells can replicate apparently without limit in culture, while remaining pluripotent. Features of ES cells are high levels of telomerase activity, a short G1 cell cycle checkpoint, and initiation of DNA replication without external stimulation. They are the only known truly immortal stem cells and most importantly maintain a normal diploid karyotype. Protocols for directed differentiation into a wide variety of cell types have been established. A practically unlimited supply of cells and a variety of options

for genetic manipulation, together with their differentiation capacity are clear advantages of ES cells as valuable tools for providing mechanistically relevant data.

However, the ethical burden of human ES cell lines will make an international acceptance of toxicological tests based on human ES cells test difficult. This is particularly the case for assays of cosmetic ingredients that are often specifically referred to in ethical discussions as one industrial use that should not be implemented if comparable methods are available (*see e.g. European Group of Ethics, opinion 22*) (*Commission, 2007*). One solution emerged very recently with the identification by Shinya Yamanaka and his colleagues of a protocol that allows induction of pluripotency in somatic cells, creating the now famous iPS cells (*Takahashi et al., 2007; Takahashi & Yamanaka, 2006*). One can foresee how iPS cells may eventually take over fully the space currently devoted to human embryonic stem cells, once comparative studies have been successfully performed. At this point, however, the wealth of data and expertise gained on embryonic stem cells over the past decade –together with the uncertainty which has not been lifted yet about the precise status of iPS cells- make it clear that both cell types deserve parallel attention.

In the ***SCR&Tox*** project, the detailed assessment of the potential of human ES cell-based tests will, therefore, provide the basis for a parallel use of genetically engineered iPS cell lines, provided they exhibit similar or even identical characteristics. Specific measures will be taken in the ***SCR&Tox*** consortium in order to design and/or implement protocols allowing for iPS reprogramming without permanent presence of the transgenes, as a way to obtain so-called "clean" cell lines more compatible with large-scale industrial use than cells that have been reprogrammed through stochastic genes integration.

Quality controls of pluripotent stem cells are well established, allowing for test standardization

A wide international effort has been made over the past years in order to precisely define relevant quality controls for pluripotent stem cell lines. The International Stem Cell Banking Initiative has also established best practice guidance for banking human embryonic stem cells (hESCs; *Crook et al., 2010*). In order to promote those cell lines to standardised use in the industry, additional controls will be unavoidable, in particular to assess variability over time in cell culture. For example the "Good Cell Culture Practice" (GCCP) principles (*Coecke et al., 2005*) were developed to promote the maintenance of high cell culture standards in order to ensure the reproducibility, relevance, acceptance for *in vitro* toxicity tests. Due to the uniqueness of pluripotent cells in general, GCCP criteria need to be expanded in order to take the specific characteristics of these cells into account.

The ***SCR&Tox*** programme will pay particular attention to the needs of a standardization of those quality controls as a basic requirement for regulatory normalization. In parallel, the

need for cost-effective measures will be carefully considered as the goal of the project implies promotion of assays to industrial-scale, i.e. taking into account feasibility as well as soundness.

Pluripotent stem cell lines give access to Human diversity for "population testing in a dish"

Even the safest drugs may cause adverse reactions in certain individuals with a specific genetic background or environmental history. A genetic component of pharmacokinetic variability was postulated more than 100 years ago in studies of patients with alkaptonuria (*Garrod, 1902*). There are now over 170 gene products known or expected to have a role in drug disposition, of which more than half are known to be polymorphic (*Katz et al., 2008*). Polymorphic enzymes account for 40 % of phase I dependent drug metabolism and today there are 69 drugs with human pharmacogenomic labels, where the polymorphic CYP2C9, CYP2C19 and CYP2D6 account for a majority of these labels (*Andersson et al., 2005; Frueh et al., 2008*) and where in many cases dose adjustments according to genotype are suggested by FDA (U.S. Food and Drug Administration) and the European Medicines Agency.

In a recent editorial, Thomas Hartung underlined the need for tackling that question (*Hartung, 2009*), writing: "*An ideal study to understand whether an agent is harmful to humans would require an extremely large number of human subjects who are representative of the diversity of humans and who are unknowingly exposed to the agent under realistic conditions*." Continuing: "*If there is any deviation from these experimental conditions, which are unrealistic and unethical, the study will provide only an approximation of the real situation —it is a model*." Within this defined framework of a model approximating the real situation, our view in the ***SCR&Tox*** consortium is that pluripotent stem cells may meet this challenge. Comparison of pluripotent stem cell lines available through collaboration with cell bank in China, Europe and the US will enable to seek differences between characterised populations and analyze the effects of genetic polymorphisms in predictive toxicology. Moreover, iPS cells derived from individuals with known susceptibilities or resistance to various drugs or diseases could eventually offer unprecedented opportunities to uncover the personal suite of genetic factors and potential epigenetic influences that relate to differences in drug responses between individuals.

Pluripotent stem cell lines provide differentiated derivatives of direct interest for toxicology testing

The variety of cellular phenotypes that pluripotent stem cells allow to reach (*Figure 4.3*) potentially offers a pharmacological research access to priority populations, particularly in the field of toxicology. Regarding major toxicological concerns, some of them on marketed drugs

that have consequently been withdrawn, numerous cellular types – such as hepatocytes, cardiomyocytes, neurons, keratinocytes and muscle cells – derived from pluripotent stem cells can find here an interest to supply an identity card of the effects of drug candidates on various tissues of the body. The ***SCR&Tox*** programme will deal in parallel with these five important lineages.

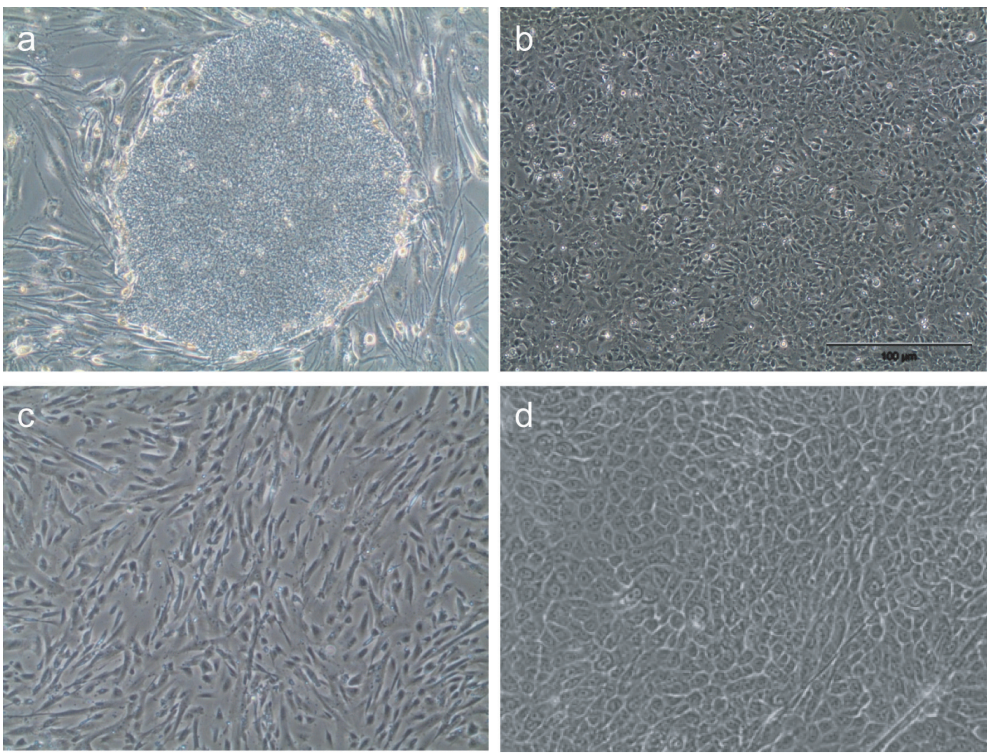

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Figure 4.3: *Human pluripotent stem cells ((a) colony of human embryonic stem cells on feeder cells) can be differentiated into many lineages including neural stem cells (b), mesodermal progenitor cells (c) and basal keratinocytes.*

➠ Liver

The research concerning the differentiation of the human ES cells into hepatocytes has considerably progressed over these past years. It is possible to obtain hepatocyte-like cells with a yield of 70% in about 20 days showing progressive commitment to definitive endoderm, hepatic induction and a maturation phase. Efficient generation of functional hepatocyte-like cells from iPSCs has also been published (*Sullivan et al., 2010*). The cells obtained by these protocols synthesise and secrete albumin, store glycogen and present transporter activity.

Hepatocytes express enzymes involved in reactions classically defined as phase I and phase II. Hepatocyte-like cells obtained following the protocol of *Hay et al.* (*2008*) express the CYP3A, although at a low level, and have a functional tolbutamide metabolism indicating presence of functional CYP2C9, whereas this drug is not metabolised by the classically used HepG2 transformed cells. Concerning phase II, the profile of sub-units which compose the enzyme glutathione transferase (GST) is comparable in hES-derived cells and human hepatocytes whereas GSTA1-1 is underexpressed in HepG2 cells (*Soderdahl et al., 2007*). Moreover, the GST is functional and responds to a cocktail of known inducers.

➠ Heart

Cardiomyocytes are readily observed in differentiating cultures of human ES (*Kehat et al., 2001; Synnergren et al., 2008*) and iPS cells (*Zhang et al., 2009*). It is easy to obtain an original precursor population of cardiomyocytes that essentially exhibits an atrial phenotype. Whether full maturation can be obtained of discrete populations of left versus right ventricular cardiomyocytes, as well as pacemaker cells, remains currently a major issue.

Besides cardiac toxicity due to modulation of signalling pathways, there is a more profound reason for cardiotoxicity as the hERG channel, which produces the IKr current, is robustly blocked by a large class of drugs (*Ameen et al., 2008*). This current has a major function in cardiac repolarization as it affects the length of the action potential and the QT interval -the duration of ventricular depolarization and subsequent repolarization. Prolongation of the QT interval on a surface electrocardiogram is officially acknowledged as a major drug safety issue (*Yap & Camm, 2003*). Today, assessment of this risk is part of the standard pre-clinical evaluation of novel drug candidates but as such, it occurs very late during drug development. A number of laboratories are, therefore, actively searching scalable, reproducible and biologically relevant novel models, preferably from an inexhaustible source. Human pluripotent stem cell-derived cardiomyocytes may have this potential.

➠ Central Nervous System

Neuroectodermal differentiation of pluripotent cells has been extremely successful, with already available protocols for both obtaining homogenously differentiated populations of self-renewable intermediate precursors and enriched cultures of terminally differentiated neurons (e.g. dopaminergic nigral neurons, striatal medium spiny GABAergic neurons, motoneurons, etc.; *Koch et al., 2009*).

Currently, there are no *in vitro* models for assessing neurotoxicity, and scientists rely on observational screens of animals to identify neurological and behavioural perturbations. In addition to providing more relevant models, neurobiologists are hopeful that cultured neurons will enable studies to be performed *in vitro* that are otherwise impractical or impossible to

achieve *in vivo*. Pluripotent stem progenies may indeed reveal promising in strengthening the identification of developmental neurotoxicants. This was illustrated by *Stummann et al. (2009)* who, following a 2-steps differentiation protocol of human ES cells into neural progenitors and neuron-like cells, and analyzing for each step the expression of selected genes, have demonstrated that methylmercury, one of the most significant outliers in the initial EST (i.e. this well-known neurotoxicant was predicted to be non embryotoxic (*Genschow et al., 2004*)), interferes mainly with the early stages of neural development. The mechanisms and concentration at which this early developmental toxicity occurs *in vitro,* correlates with the clinical data (*Stummann et al., 2009*).

➠ Skin

With the progressive implementation of REACH and the ban on animal testing for cosmetics already initiated and fully effective in 2013, *in vitro* skin models are arousing a marked interest not only from the pharmaceutical but also from the chemical and cosmetics industries (*Rovida & Hartung, 2009*).

The differentiation of human ES cells into fairly pure keratinocyte populations has already been described (*Guenou et al., 2009*). After 10 days of culture on polycarbonate inserts at the air-liquid interface, these hESCs-derived keratinocytes demonstrated their functionality by forming a pluristratified epidermis displaying expression of relevant markers in the different layers. The development of sophisticated pigmented epidermis representative of the different phototypes, by co-culture with melanocytes, and the transposition of all the human ES protocols to iPS are some of the next challenges in this field. Such pluripotent stem cells-derived tridimensional skin models could confer several advantages in terms of quantity, standardization and representation of genetic polymorphisms compared to the presently available reconstructed epidermis -obtained with human normal keratinocytes resulting from plastic surgery or neonatal tissues- that are used to predict different drug parameters.

➠ Muscle

Skeletal muscle full differentiation of pluripotent stem cells has, up to now, remained difficult. However, self-renewable intermediate precursors of the mesodermal lineage (exhibiting a number of phenotypic traits of mesenchymal stem cells) are readily obtained at homogeneity (*Mateizel et al., 2008*) and have demonstrated instrumental at least in one case of screening for myopathic toxicity.

Cerivastatin is an aknowledged myotoxicant that has been withdrawn from market in 2001 due to reports of fatal rhabdomyolysis leading to death in 52 cases. Interestingly, gene polymorphism and drug interactions (*Niemi, 2010*) interfere with this toxic effect. Exploring the potential of pluripotent stem cells to model statin myotoxicity would thus be of main interest.

Pluripotent stem cells are amenable to medium and high throughput industrial-scale technologies

The miniaturization of cell-based assays is inseparable from the implementation of high-throughput screening technologies requested by promotion of toxicology testing at an industrial scale. Pluripotent stem cells, including of human origin, have already been used in such formats, thus opening the path toward industrial use. At least three already published reports demonstrate that pluripotent stem cells can be used for drug screening on HTS platform (*Chen et al., 2009; Desbordes et al., 2008; Zhu et al., 2009*). Three of the partners of the ***SCR&Tox*** programme have already successfully experimented – though not published, yet – with human ES cell-derived derivatives directly on proprietary HTS platforms for various purposes, including drug discovery through screening of compounds libraries, mechanistic approaches using selected samples of siRNA or vectorised cDNA to perform functional genomic screens, and searching for differentiation factors.

Concluding remark

Altogether, this rapid review of the information on which we base the ***SCR&Tox*** programme shows that knowledge of fundamental mechanisms that govern pluripotent stem cells amplification is very rapidly progressing. Guided differentiation toward derivatives of interest for toxicology testing is based on more and more refined and relevant protocols. Technologies for analysing, engineering and screening those biological resources are already largely developed. Altogether, this will help to overcome eventual difficulties in designing and implementing cell-based high throughput assays for toxicity up to the industrial scale.

4.2.4 Approach

In order to develop our working hypothesis and demonstrate the potential of pluripotent stem cells derived systems in developing high-predictivity *in vitro* models for toxicology testing, ***SCR&Tox*** assembles partners with strong expertise in skills ranging from pluripotent stem cells biology to innovative technology with an industrial perspective and has been built following the described organization. From a scientific point of view, our approach is articulated upon the 4 following parts that are illustrated in *Figure 4.4*.

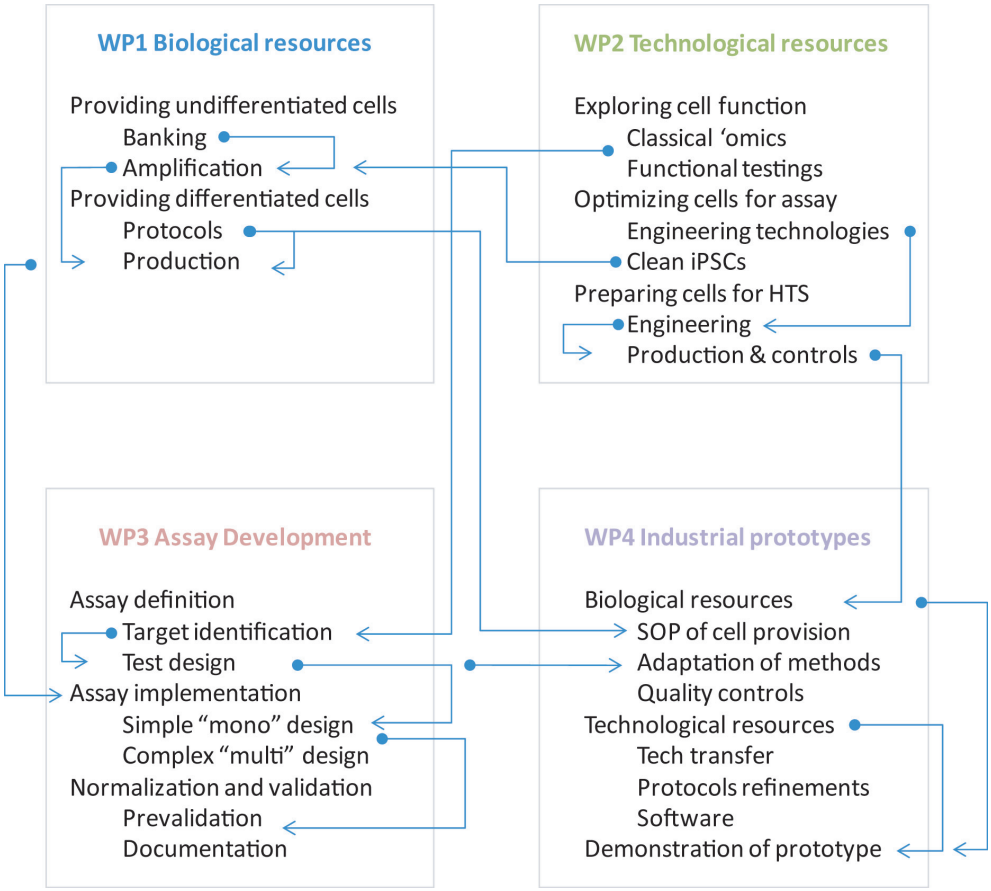

Figure 4.4 *Work package structure, tasks and interactions.*

Success of scientific aims is supported by 2 additional work packages: training and dissemination (work package 5) and project management and coordination (work package 6).

Work package 1: Biological resources

The first part of the ***SCR&Tox*** Project is dedicated to the provision of biological resources required for assaying toxicity pathways. Human embryonic stem cells and induced pluripotent stem cells are the two basic resources. Their use requests mastering and orienting their major attributes – self renewal and pluripotency – toward their specific use in testing toxicity of chemicals, namely their diversity of origins, scalability at the undifferentiated stage and pluripotency, in order to create the conditions for production in needed quantity and quality.

➠ Providing undifferentiated pluripotent stem cells

Banking of cell lines at the undifferentiated stage: Diversity is a major advantage of pluripotent stem cell lines, as it allows exploring the impact of genetic polymorphisms on the responses to chemical in Human (*Pouton and Haynes, 2007*). The first task of the ***SCR&Tox*** programme is to master that capacity by managing banking of all cell lines deemed necessary for the project. The first phase thus consists of preparing a "working cell bank" of all hES and iPS cell lines required for the project, with access for the other consortia.

Design of automated methods and standardization for scaled-up production of pluripotent cells: Mastering pluripotent cells production scale up appears to us as an important challenge for the final transfer of an assay on industrial platforms that may eventually require billions of cells to be produced from each cell line. Once available in the working cell bank, undifferentiated cells will be amenable to large-scale production (over 100 million cells). Conditions for scalable cell culture of human ES cells are available at several partner sites and will be adapted to iPS cells and evaluated with respect to achievable scale and cell quality.

➠ Providing differentiated derivatives of pluripotent stem cells

Establishment of protocols for coaxing cells along the 5 lineages of interest: Three ***SCR&Tox*** partners have been among the most successful teams in that domain, with protocols published and patented for a diversity of lineages including liver, CNS, heart and epidermis. Optimization of these existing protocols is required in order to fulfil specifically the needs of large-scale screening assays, with particular focus on reproducibility of the protocols –including QC for each.

Production of "ready-to-screen" cell kits and plates: Frozen banks of differentiated derivatives will be established for further use in the assay development phase (WP3). These cells will, in parallel, be made available to all relevant consortia of the **SEURAT-1** Research Initiative, in particular for analyzing biomarkers and endpoints of toxicity pathways. It is expected that most cell phenotypes obtained at sufficient homogeneity and quantity will be amenable to plating, at least in 96 well-plate formats. We will make use of these to prepare cells in a "ready-to-screen" format (*Figure 4.5*).

Figure 4.5 *Automated cell culture system for massive amplification of pluripotent stem cells and their derivatives.*

➠ Risks and contingency plans:

One risk is a complete impossibility to scale-up production of undifferentiated cells. It would leave us with the capacity to produce cells for the programme using time-consuming but classical non-automated scale-up techniques. Concerning the establishment of protocols for coaxing cells along the 5 lineages of interest, protocols are either fully available, or else, are at close reach for most lineages and stages of differentiation. A risk of failure specifically exists, however, for myoblasts, the differentiation of which has remained elusive. Mesodermal precursors have revealed, however, instrumental in replicating myopathic toxicity. Contingency plan would, therefore, just reduce the number of phenotypes assayed from 10 to 9.

Work package 2: Technological resources

The second part of the ***SCR&Tox*** project aims at providing all technologies and methodologies required for developing efficient cell-based assays seeking quantitative assessment of biomarkers associated to toxicity pathways.

➠ Technologies for profiling cell and gene expression and exploring cell functions

Implementation of classical “omics” to demonstrate the value of stem cells derivatives for current approaches in the exploration of signalling pathways: Three main technologies, namely

transcriptomic, proteomic and metabonomic, will be implemented in order to obtain, at the molecular level, a full multi-parametric documentation of the cells to be assayed (*Robertson, 2005*). This is all the more important as the exploration of toxicity pathways is unavoidably quantitative, i.e. by definition the signalling pathways exist and are functional, and exposure to toxicants are mere perturbations.

Functional testing: Design and implementation of functional genomics (cDNAs or siRNAs) and "interactomics" methodologies will be used to dynamically analyse stem cell derivatives. Functional approaches will be especially useful in addition to classical 'omics for seeking and characterizing toxicity pathways, as well as in subsequent steps using biomarkers for identifying toxicants that would perturb specifically those pathways (*Winkler et al., 2009*). This includes the characterization of electrogenic properties of stem cell derivatives with neural, cardiac or muscle phenotypes through automated screening with bioelectronic multielectrode-microarrays and multiwell-microcavity-arrays (*Kloss et al., 2008*).

➠ Design and implementation of new cell engineering methodologies for providing them with properties of interest for assay design

Exploration and implementation of two main technologies newly-designed by ***SCR&Tox*** partners: (i) First, genome engineering methodology using specifically designed DNA endonucleases ("meganucleases") (*Arnould et al., 2006*). This part of the programme will aim at developing and implementing molecular "landing pads" (i.e. meganuclease-based tools that induce targeted integration of any chosen transgene in a chosen single location in the human genome) for subsequent flexible uses and that are not silenced in human pluripotent stem cells or in the downstream lineages. (ii) Second, a protein production and transfer methodology using VSVG-decorated vesicles ("gesicles").

Implementation of a genetic reprogrammation methodology that leaves no permanent scar in the genome of iPS cell lines: Three technologies will be assayed for obtaining a method for somatic cell reprogrammation that will leave no permanent scar in the iPS cells genome: (i) recombination at the meganuclease-introduced landing pads of a pluri-cistronic construct that comprises genes necessary for reprogrammation, (ii) optimization of the reprogrammation system by combining meganucleases with gesicles, (iii) implementation of a different strategy relying on the cre/lox system.

➠ Optimization and production of stem cells derivatives for HTS

Engineering cells with constructs facilitating cell phenotype selection or promotion. This programme will have two phases, as partners will implement technologies for cell engineering in parallel and identify gene constructs of interest for selecting cells exhibiting a certain phenotype or else promoting differentiation toward a discrete lineage. Transgenes/cassettes

candidate will be designed and tested to be plugged into the above-mentioned landing pads to improve the derivation of cell/tissue chosen along the relevant types as implemented in WP3. In parallel, a systematic and robust method will be developed using a custom engineered meganuclease to knock-in a trackable/purifiable marker gene, in order to allow robust efficient enrichment of differentiating cells in the desired precursor type(s). Gene constructs will essentially be of two types: (i) a gene encoding a selection marker will be driven by a promoter derived from that of a gene, the expression of which is triggered specifically at the stage and type of differentiation desired or (ii) a gene encoding a transcription factor known to be instrumental for cell differentiation in the desired phenotype, or else a discretely efficient microRNA will be placed under the control of an inducible promoter. Conversely, gesicle-transfer of the transcription factor protein may be used for temporary action, if deemed useful.

Production of optimised ready-to-screen cells banks and plates for HTS conditions *(Figure 4.5)*. Cells of 5 phenotypes and at least two different stages of differentiation will be produced in sufficient amounts for the planned use in assay development (WP3) and stored conveniently. Those biological resources will be readily made available to all consortia of the **SEURAT-1** Research Initiative.

➠ Risks and contingency plans

Scar-free reprogrammation is probably the most risky part of the entire project, justifying our parallel attempts at different protocols. In case of failure, plan B relies on the episomal expression of reprogramming genes.

Work package 3: Assay development

Starting at half-term of the programme, this part aims at integrating data from all consortia of the **SEURAT-1** Research Initiative in order to make use of the obtained biological and technological resources for developing, “at bench scale”, at least one cell-based assay of a toxicity pathway. After checking for relevance, i.e. observing the test signalling pathway in pluripotent stem cells derivatives and its perturbation by a provided selected reference toxicant in the different assayable cell phenotypes, a test assay will be designed and implemented at low scale, up to results allowing pre-validation.

➠ Establishing the existence and function in pluripotent stem cells derivatives of the toxicity pathway to be further used as a test

This will include: (i) The precise characterization of the toxicity pathway in the different stem cells derivatives in order to identify the read-out(s) that will be used for assessing quantitatively the effects of toxicants. (ii) The design of specific gene constructs and development of measuring

methodologies for identifying effects of perturbators on the tested toxicity pathway. (iii) The exploration of perturbations of the tested toxicity pathway in the presence of a reference toxicant, using classical and functional genomic and proteomic, as well as bioelectronic methods whenever relevant.

➠ Design, development and implementation of an assay at "academic scale" as proof of concept

Screens of a set of toxicants selected and prepared by the other consortia and the Data Warehouse will be performed in order to validate the developed assay for studying toxicity-related perturbations of the tested pathway in conditions varying (i) doses, duration and exposure regimen and (ii) phenotypes and genotypes as proof of principle and in order to fulfil ECVAMs criteria for entering into pre-validation.

An additional assay will be performed in the search for the impact of the hepatic production of chemicals metabolites, using human pluripotent stem cells-derived heaptocytes to produce conditioned medium when treated with the tested toxicants, and secondarily assaying application of this medium potentially containing toxicant metabolites on the other cell phenotypes.

➠ Prevalidation of the assay and normalization and validation process

This part includes: (i) The normalization of the developed assay (description of the mechanistic basis of the test method and the selected endpoints, standard operation procedures including acceptance criteria); and (ii) the Establishment of templates and datasets allowing for statistical evaluation and subsequent testing of intra- and inter-laboratory variability opening the regulatory path for validation.

➠ Risks and contingency plans

In the event of the absence of expression/function of the selected toxicity pathway in the different stem cell derivatives under study, a second and potentially a third pathway would be taken in the list established by the **SEURAT-1** Research Initiative.

A high intra-laboratory variability due to variability of cells would require further standardisation of the biological resources in WP1.

Work package 4: Testing a prototype assay for a toxicity pathway on an industrial platform

This part will operate the promotion of the selected cell-based assay up to the industrial scale. It will require technology transfer to the two CRO's of our consortium as well as methodological adaptation and refinement in order to reach the final step of the programme, which is the proof of concept that pluripotent stem cells derivatives can prove to be instrumental for testing a toxicology pathway in a relevant, efficient, extended and normalised assay on an industrial platform.

➠ Establishing standard operating procedures for adaptation of biological resources to an assay on industrial platforms

This part consists of (i) the validation of standard operating procedures for cell differentiation in an industrial context, and (ii) the implementation and validation of biological resources on the industrial platforms and establishment of standard operating procedures for Quality Controls of biological parameters.

➠ Transferring technologies and methodologies of the developed assay for implementation on industrial platforms

This implies technology transfer, refinement of protocols, as well as design and implementation of software tools required for analysis.

➠ Demonstration of the prototype assay on industrial HTS platforms

Demonstration of the robustness of the assay for varying test conditions: In order to justify mobilising the resources of national and international agencies in the acceptance of the developed assay methods, data must be available demonstrating the ability of the assay system to distinguish positive and negative responses in a reproducible way and with an acceptable rate of false negatives and false positives. For this purpose a first test will be made of the robustness of the assay, using the reference toxicant as a positive control and quantifying Z' factors (*Zhang et al., 1999*) for each of the two relevant test conditions (i.e. single dose exposure and repeated dose exposure over two weeks).

Demonstration of the specificity, sensitivity and reproducibility of the assay for all test conditions: Specificity, sensitivity and reproducibility of the assay will secondarily be tested against all test conditions (i.e. varying doses, exposures, phenotypes and genotypes) at the two sites. A well-sized library of test compounds will be used for that purpose at the two sites in parallel and in triplicates. This will allow determining the specificity of the test (through a list

of demonstrated pertubators of the toxicity pathway), its sensitivity (by showing quantitatively differences between the different effective compounds) and reproducibility both on the same platform and between the two.

➠ Risks and contingency plans

This work package aiming at the scaled-up implementation of a validated assay on industrial platforms shows little foreseeable risk of a complete roadblock.

Work package 5: Training and Dissemination

SCR&Tox training and dissemination programme aims essentially at helping end-users – pharmaceutical and cosmetic industry as well as members of laboratories that participate to other consortia of the **SEURAT-1** Research Initiative – obtain expertise and, whenever relevant, technology transfer in order to be prepared for implementing or being able to regulate the new paradigm for predictive toxicology based upon stem cell-based assays.

In addition, ***SCR&Tox*** will provide training for the use of its biological resources (pluripotent stem cells and derivatives) and technological resources (in particular robotic systems) to members of consortia belonging to the **SEURAT-1** Research Initiative, in order to help them implement those cells and technologies in their own part of the common project.

This will be conducted through 2-3 month immersion in ***SCR&Tox*** laboratories, as well as longer term technology transfer designed on case to case basis.

In addition, annual workshops will be dedicated to provide members of European regulatory bodies with training to the scientific and technical bases as well as up-to-date information on cell-based assays in predictive toxicology.

Work package 6: Project management and coordination (WP6)

The main objective here is to ensure the effective management and coordination of the project in order to guarantee a high efficiency and high quality execution of the work. Besides the usual and contractual administrative tasks, the project management will focus as priority on monitoring complex aspects related to network's specificity such as information hub, database management and gender equality promotion. Furthermore, a comprehensive set of activities has been included to coordinate the communication activities inside and outside the consortium, and within the general framework of the **SEURAT-1** Research Initiative.

Management objectives can be summarised as follows:

- ➠ To act as the interface between the ***SCR&Tox*** consortium and the European

Commission and other funding bodies, respectively.

➠ To ensure that all actions are performed correctly and within the rules and regulations established by the European Commission and in the consortium agreement including financial and legal management and to ensure that the received funds are correctly distributed and accounted for.

➠ To ensure the work and tasks are performed on time, within budget and to the highest quality and create an early warning system.

➠ To keep each partner, including the Commission and other funding bodies, fully informed about the project status, scientific issues, the work planning (adjustments) and all other issues which are important and relevant to partners in order to obtain maximum transparency for all involved and achieve synergy of the cooperation; to ensure that all partners are informed of all important and impacting information that can influence the outcome of the project.

➠ To assure efficient communication and knowledge transfer within the rules and regulations established by the European Commission in the framework of the **SEURAT-1** Research Initiative.

Overall, this structure aims at creating a supportive, collaborative culture with reduced risk of mistakes, redundancy, quick problem solving, good decision making, increased worker independence, productivity and services, all adding up to keep the project at critical mass. It involves key stakeholders ensuring an inventory of competence matching responsibilities and participant capabilities with a conscious strategy to get the right knowledge to the right people at the right time. This results in junior and senior member development, and strategies for proper information usage, guaranteeing a complete return on the intellectual capital, and ensuring that the potential to disseminate or intellectually protect any generated information is constantly assessed.

4.2.5 Innovation

A particularly innovative aspect of our programme is the emphasis on the full use and mastering of all the DIVERSITY and VERSATILITY offered by those cell lines, for analysis of multiple cell phenotypes (in 5 different organs of interest for toxicology), multiple conditions of exposure (single *vs* repeated –low *vs* high doses), multiple genotypes (offering a stochastic approach of human polymorphism *in vitro*) and multiple approaches (both in terms of analyses, in particular with “functional ‘omics”, and engineering to optimise and standardise). Most importantly newest cell biological approaches and molecular biological techniques will be undertaken for the successful cellular differentiation therapy leading to target cells relevant for assessing systemic repeated dose toxicity.

To achieve this ***SCR&Tox*** has assembled an international team of researchers that have collectively an enormous past record of innovation in the industrial sector as assessed by a large number of patents in highly competitive domains such as pluripotent stem cell production (including bioreactors and specific culture media) and differentiation (in most of the five lineages explored in parallel by ***SCR&Tox***), cell engineering at the genome and protein levels (in particular for the two newly developed technologies implemented in the programme, meganucleases and gesicles) as well as for bioelectronics in microarray and microcavity array. CRO partners are among the world leaders in the field of contract toxicology testing. It is foreseen that the ***SCR&Tox*** Project will continue on those paths and be highly successful in bringing new IP and industrial position to its partners.

Positioning of the different partners along the scientific continuum in the ***SCR&Tox*** Project is displayed in the scheme below (*Figure 4.6*).

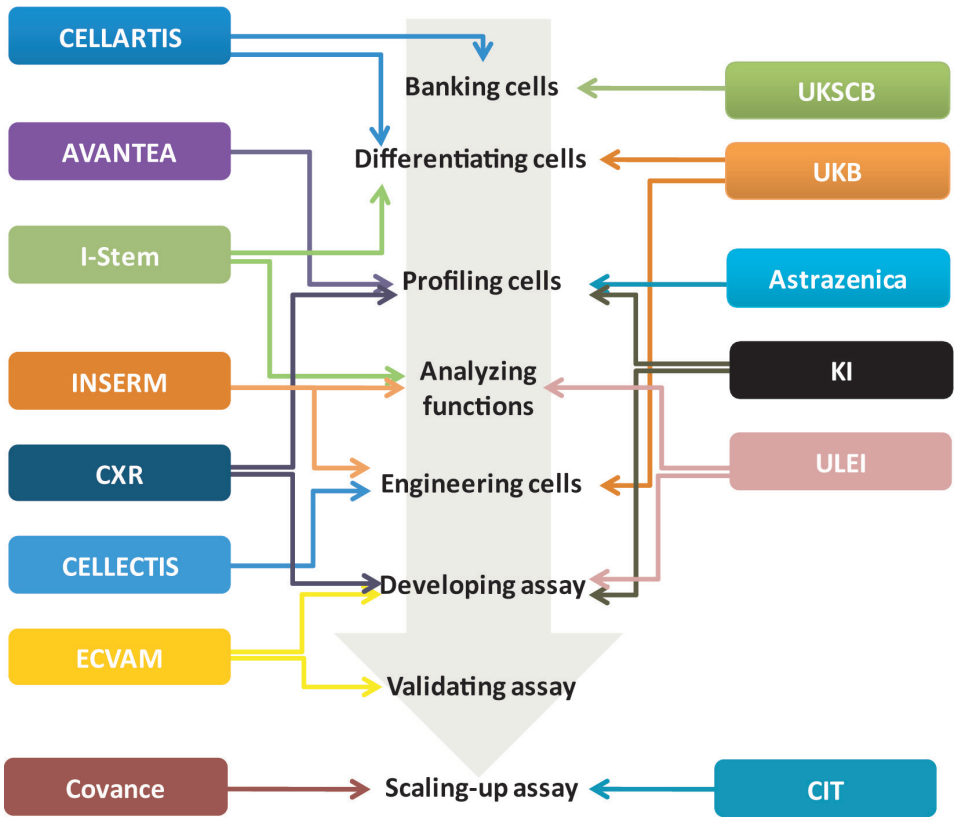

Figure 4.6 *Main focus of participation of the SCR&Tox partners, showing their complementarity along the scientific/technique continuum of the Project (along the middle arrow, steps in black). Each partner is represented by one colour; a maximum of two main areas of involvement are indicated by arrows.*

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4.3 HeMiBio: Hepatic Microfluidic Bioreactor

Catherine Verfaillie[1]

4.3.1 Introduction

Refinement, Reduction and Replacement of animal usage in toxicity tests, the so-called 3Rs principle, is of particular importance for the implementation of relevant EU policies, such as the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Regulation (EC1907/2007) or the 7th amendment to the Cosmetics Directive (Directive 2003/15/EC amending Council Directive 76/768/EEC). Although multiple projects have been funded by the EC aimed at implementing the 3Rs principle in toxicity testing, the assessment of toxic effects of (sub)chronic exposure still requires a high consumption of animals as no alternative methods are yet available for these *in vivo* tests. Aside from these ethical considerations, there is a great need for suitable human cells for toxicity testing, due to the often poor concordance between animal models and humans.

In ***HeMiBio***, we propose to *generate a liver-simulating device mimicking the complex structure (Figure 4.7) and function of the human liver*. The device will reproduce the interactions between hepatocytes and non-parenchymal liver cells (hepatic stellate cells (HSC), hepatic sinusoidal endothelial cells (HSEC), and hepatic Kupffer cells) for over 1 month *in vitro*. Such a Hepatic Microfluidic Bioreactor could serve to test the effects of repeated exposure to chemicals, including cosmetic ingredients. To create such a device, the cellular components of the liver need to be viable for over 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 usable for (iv) screening for 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 criteria set forth above. With increasing complexity, hepatocyte function is maintained

[1] on behalf of the ***HeMiBio*** consortium

over extended periods of time, whereas the less complex culture systems are more amenable for studying the mechanisms that control maintenance of cellular function.

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

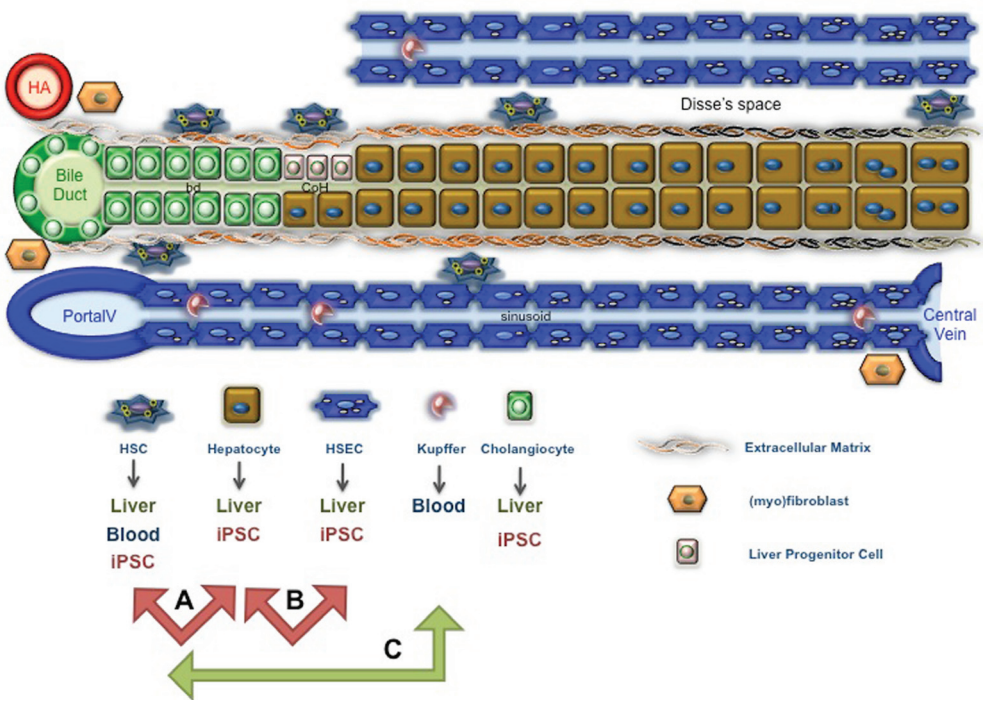

Figure 4.7 *Schematic representation of a liver sinusoid (adapted from: Dollé et al., 2010).*

The underlying hypothesis for successful creation of a 3D liver-simulating device suitable for repeated dose toxicity testing 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 4.7* may not be required, the very short distance cellular interactions shown between (A) hepatocytes-HSEC and (B) hepatocytes-HSC cells will be necessary for maintaining the functional state of the three cell types, (C) and presence of monocytes/Kupffer cells will be required to fully assess drug toxicity.

4.3.2 Objectives

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

- ***HeMiBio*** will develop tools to engineer *three different liver cell types* (hepatocytes, HSEC and HSC) generated from iPS cells (or expanded using the upcyte® technology) to be used in the hepatic bioreactor. This should allow the specific and spatially defined enrichment of the different liver cell components; and to non-invasively and in real-time assess the differentiation state of the hepatocytes and the non-parenchymal cells.

- ***HeMiBio*** will incorporate *molecular sensors* 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 (first objective). In addition, innovative *electro-chemical sensors*, such as ion-selective electrodes, will be integrated in the 3D-bioreactors to allow assessment of function (e.g. oxygen uptake, ammonium, and glucose concentrations), and also the continuous assessment of cell integrity (e.g. by measurement of potassium, and enzyme release due to cell death).

- ***HeMiBio*** will develop a *2D-bioreactor* for the efficient isolation of differentiated iPS cells 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).

➠ ***HeMiBio*** will generate 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 above-mentioned engineered cells and sensors under 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.

➠ ***HeMiBio*** will provide proof-of-principle that a liver-simulating device can *recreate the toxicity profile in vitro* of toxins with a known *in vivo* toxicity profile over a minimum of one month, including the barrier function of the liver and the effect of inflammatory (and immune) cells in this process.

➠ ***HeMiBio*** will assess the *molecular, functional and metabolic phenotype* of the hepatocellular, HSEC and HSC components at all stages of bioreactor development, and compare this with that of cells isolated fresh from human livers.

4.3.3 State of the Art

The adult liver weighs 1.4-1.6 kg representing ~ 2.5 % of body weight. Incoming blood arrives via the portal vein and hepatic artery and leaves through the hepatic vein. The liver sits at the junction between the digestive tract and the rest of the body and consequently is a key player in metabolism, including processing dietary amino acids, carbohydrates, lipids, and vitamins, protein synthesis, and detoxification and excretion of toxins. The liver consists for > 65 % of hepatocytes, responsible for most of the liver functions, but also contains other cell types, including endothelial, stellate and Kupffer cells (*Table 4.1*), all with highly specialised functions. To mimic liver function, many increasingly more complex and clinically relevant approaches can be used, starting from plasma membrane vesicles or the microsomal fraction of hepatocytes, malignant hepatocyte cell lines, cultured primary hepatocytes, liver slices, perfused liver lobules, and ultimately the human liver itself (reviewed in *Dunn et al., 1991*, and *Abu-Absi et al., 2002*). Due to shortage of human livers, however, cultured primary hepatocytes and perfused liver lobules or liver slices are impractical even if they may be quite predictive and clinically relevant. In addition, primary hepatocytes rapidly de-differentiate under standard conditions (*Dunn et al., 1991; Abu-Absi et al., 2002*).

Table 4.1 *Cellular composition of the liver.*

Tissue Compartment		% volume	% cells
Hepatocytes		77.8 ± 1.2	65 – 70
Sinusoidal cells	Total	6.3 ± 0.5	30 - 35
	Endothelial cells	2.8 ± 0.2	14.8 ± 0.9
	Stellate cells	2.1 ± 0.3	7.1 ± 0.5
	Kupffer cells	1.4 ± 0.2	10.5 ± 0.9
	NK/Lymphocytes	n.d.	n.d.
Intercellular space		15.9 ± 0.8	0

Hence what is needed for the cosmetics and pharmaceutical industry are innovative culture systems that incorporate hepatocytes as well as non-parenchymal liver cells, derived from an expandable/renewable cell source. These co-cultures will allow induction and maintenance of mature hepatocyte, HSEC and HSC function, while creating a bioreactor that can provide clinically relevant information on drug and chemical clearance and toxicity, allowing testing of repeated dose toxicity for several weeks to ultimately months. However, no reactor has yet been created that can indeed fulfil all criteria set forth above.

The need for hepatic bioreactors to test repeated dose toxicity of cosmetics and pharmaceuticals

Even following extensive selection of chemicals for lead compounds more than 10,000 compounds are commonly tested to produce a single compound that reaches the market. The average cost of generating a drug that makes into the market was estimated to be around $3 billion in 2000, and only one out of three drugs that does reach the market is profitable. The pharmaceutical industry uses in general efficacy tests in animals to identify drugs with possible clinical efficacy. Unfortunately, although candidates are identified with excellent properties in animal models in this manner, they demonstrate no efficacy in humans. Two of the major problems have been bioavailability and toxicity, both of which can be related at least in part to metabolism. Hence, one of the major hurdles facing the pharmaceutical industry is to develop better assays to predict bioavailability as well as toxicity.

Bioartificial liver (BAL) devices are under development and are being tested in a clinical context with the purpose of replacing hepatic function in patients with acute liver failure (*Kobayashi, 2009*). Their use for toxicity screening of new chemical entities, however, is in its infancy. However, successful development of small-scale laboratory systems based on human cells would be very promising for a variety of research purposes, including investigations on xenobiotic metabolism, hepatotoxicity, liver function and liver disease (*Dash et al., 2009*).

The urgent need for such new *in vitro* systems mainly stems from the overall poor correlation between animal and human liver toxicity, the high attrition rate of new chemical entities and a number of scientific, legal, ethical and economic difficulties, in particular encountered in Europe in using experimental animals. The more sophisticated BAL systems are expected to reflect more appropriately the *in vivo* toxic responses of pharmaceuticals, cosmetic ingredients, etc. In particular, phase I and II xenobiotic biotransformation, drug transporter function and a broad range of tissue responses are much more *in vivo*-like in BAL systems in comparison with conventional cultures of primary hepatocytes. Xenobiotics are usually lipophilic and undergo biotransformation in the liver, yielding more hydrophilic and thus easily excretable metabolites. In sharp contrast to this detoxification scenario, xenobiotics can be activated and can thus become toxic (*Boelsterli, 2002*).

Hepatocyte functionality in BAL systems is improved by taking into consideration the many factors that exert beneficial effects on xenobiotic biotransformation, namely the establishment of both cell-cell interactions and cell-extracellular matrix contacts, and a number of soluble factors secreted by non-hepatocytic liver cells (*Dash et al., 2009*). Partners of ***HeMiBio*** showed that the 3D-assembly of liver organoids from rat hepatocytes, endothelial cells and fibroblasts maintained liver-specific function including albumin production and cytochrome P450 (CYP450) activity for over 50 days in culture (*Nahmias et al., 2006; Kidambi et al., 2009*). Others created micropatterns of hepatocytes and 3T3-J2 fibroblasts in which hepatocyte function was shown to be maintained through a combination of cell-cell contact and diffusible substances (*Bhatia & Chen, 1999; Bhatia et al., 1997*). Stellate cells were also shown to stabilise hepatic urea and albumin secretion for up to a month in culture (*Morin & Normand, 1986; Morin et al., 1988*). Moreover, interactions between endothelial cells and fibroblasts/ stellate cells are thought to play a critical role in the development of the mature hepatocyte phenotype (*Matsumoto et al., 2001*).

Possible designs for hepatic bioreactors to test repeated dose toxicity of cosmetics and pharmaceuticals

Microfluidics allow for the dynamic control of the cellular microenvironment at the microscale which cannot be achieved in static culture (*Andersson & van den Berg, 2004*). Flat-plate bioreactors have been used to study hepatocyte function and differentiation by several groups, including one of the partners of HeMiBio. Using this model, it was shown that reducing shear flow reduces mechanical damage, but invokes the need for increasing the oxygen concentration by incorporating a membrane oxygenator in order to better preserve hepatocyte function. Another strategy is to protect cells from shear by seeding them in groves (*Park et al., 2005*) or microwells (*Khademhosseini et al., 2005*). Moreover, by providing a stable oxygen and hormone gradient *in vitro*, cultured hepatocytes show aspects of zonal differentiation (*Allen & Bhatia, 2003*).

The packed-bed reactor is a variant in which hepatocyte aggregates are perfused in an environment that allows for 3D-organisation (*Strain & Neuberger, 2002; Powers et al., 2002*). The integration of heterotypic cell-cell interactions is an additional level of complexity required for capturing the function of the *in vivo* liver. One promising set-up is the LiverChip, designed to mimic physiological shear (*Domansky et al., 2010*). The cells formed liver-like tissue structures including hepatic, endothelial, and stellate components and demonstrated albumin secretion and CYP450 activity. The LiverChip was more recently used to study drug toxicity. Another group showed similar organisation of hepatic cells, HSC and HSEC lines in a radial-flow bioreactor. Hepatocytes seeded in this reactor maintain albumin and urea secretion as well as CYP450 activity for two weeks *in vitro* (*Powers et al., 2002; Ohshima et al., 1997; Murtas et al., 2005; Li et al., 1993; Sivaraman et al., 2005; Basu et al., 2006*).

Source of cells for hepatic bioreactors to test repeated dose toxicity of cosmetics and pharmaceuticals

Although it is in theory possible to isolate hepatocytes, stellate cells and endothelial cells from human liver tissue to be used to assemble hepatic bioreactors, shortage of human tissue and poor control over the quality and function of primary cells, makes this option less desirable.

One approach to overcome the shortage of cells, is to expand the small number of cells available from human liver. However, in most instances, this leads to de-differentiation of the cell populations. However, the upcyte® technology available via one of the partners of ***HeMiBio***, may allow expansion of hepatocytes, and we hypothesise perhaps also HSEC and HSC, without significant de-differentiation, for 20 population doublings. This cell source will then also be further evaluated in ***HeMiBio***.

An alternative to primary tissue-derived cells, are stem cells that could constitute a renewable source of hepatocytes suitable for toxicity studies. Possible sources of cells are liver-derived progenitors, although very little information is available regarding the phenotype and no good methods for prospective isolation and *in vitro* expansion and differentiation of such progenitors are known (*Sancho-Bru et al., 2009*). An alternative is more pluripotent stem cells such as ES cells. Mouse ES cells, derived from the inner cell mass of the blastocyst (*Nichols et al., 1998*) are pluripotent: they generate all somatic and germ line cell types, including hepatocytes various mesodermal cells, and expand without obvious senescence. Human ES cells were first isolated in 1998 (*Thomson et al., 1998*), and have similar pluripotent features.

The recent evidence that adult cells may be reprogrammed to cells with ES cells features opens also the possibility of generating ES cells-like cells representing individuals with different toxicity and metabolic profile (*Terada et al., 2002; Taranger et al., 2005*). In 2006-7 Yamanaka's group demonstrated that it is indeed possible to induce pluripotent characteristics in somatic mouse cells by introducing four transcription factors (*Takahashi & Yamanaka, 2006; Okita et al., 2007*). In 2007, the same was achieved with human cells using a different

set of factors (*Takahashi et al., 2007; Yu et al., 2007*). iPS cells are highly similar to ES cells and generate cardiomyocyte-, neuron-, endothelial-, and hepatocyte-like cells, among others, upon directed lineage differentiation. Although cells with some functional features of hepatocytes have been generated from ES cells/iPS cells, fully mature hepatocytes are yet to be generated, and may well require co-culture with HSC and HSEC. Ideally, one would create HSC and HSEC from the same ES cells/iPS cells line, to be used in a hepatic bioreactor. One recent study described the generation of HSEC from mES cells (*Nonaka et al., 2007*) and the same research group studied the developmental steps in HSEC differentiation during mouse embryogenesis (*Nonaka et al., 2007*).

One of the ***HeMiBio*** partners has demonstrated that during induction of ES cells and iPS cells to hepatocyte-like cells, multiple mesodermal cell types are also present in iPS cells progeny, including (progenitors for) HSEC and HSC (*Roelandt et al., 2010*). Despite the presence of these mesodermal cells, hepatocytes generated from ES cells /iPS cells are still immature and the final differentiated product from human iPS cells contains large islands of immature a- fetoprotein-/Albumin+ cells, and approximately 5% more mature a-fetoprotein-/Albumin+, Phosphoenolpyruvate carboxykinase, CYP3A4+, and G6PC+ cells (*Figure 4.8*). Cells have inducible CYP450 activity, produce urea and albumin, and store glycogen, albeit at levels lower than primary hepatocytes.

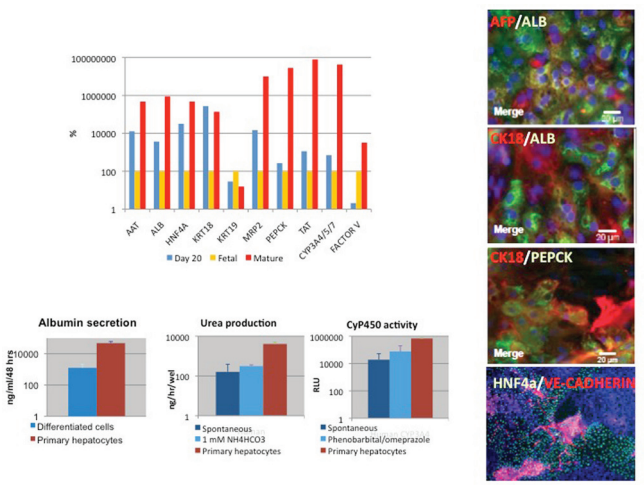

Figure 4.8 *Differentiation of human ES cells to hepatic cells. Shown is: upper left panel: transcript levels of ES cells-progeny compared with fetal and adult primary human hepatocytes; lower left panels: functional assessment of hepatic progeny from human ES cells compared with human primary hepatocytes; right panels: immunofluorescence analysis of hepatic progeny from human ES cells identifying albumin (ALB), α-fetoprotein (AFP), cytokeratin (CK)18, phospho-enolpyruvate carboxykinase (PEPCK) immature and mature hepatocytes and vascular-endothelial (VE)-cadherin-positive endothelial cells (adapted from: Roelandt et al., 2010).*

4.3.4 Approach

Although it thus appears that all cellular components present in a hepatic sinusoid, or their precursors, may be present in the ES cells /iPS cells progeny, they do not provide the necessary cross-talk to allow their full maturation. It is therefore unlikely that sequential bioreactors connected by microfluidic systems will allow maturation of the hepatocytes to fully mature cells. Likewise, it is quite uncertain that the typical fenestrated endothelial barrier will be created unless the hepatocytes and endothelial cells are capable of making physical connections as in the liver sinusoid. In ***HeMiBio*** we therefore wish to isolate the three cell components separately and then reassemble them allowing physical interactions between all three, which should allow full maturation of the cells. Likewise such configuration will likely also allow maintenance of differentiated function for long periods.

HeMiBio wishes to create a prototype microfluidic device containing hepatocytes and non-parenchymal liver cells for repeated toxicity testing of cosmetic ingredients and pharmaceuticals, to ultimately eliminate the need for laboratory animals in toxicity testing. The overall structure of HeMiBio including tasks of the different work packages are given in *Figure 4.9*. Human iPS cells, cultured under standard differentiation protocols, yielding immature hepatocytes and cells with some features of HSC and HSEC, will be used, as they can be generated from individuals with different metabolic and toxicity profiles, and expanded for more than 100 population doublings, thus representing a steady source of cells for liver-simulating devices. As an alternative, we will test if primary hepatocytes, HSC and HSEC can be expanded using Medicyte's proprietary technique for 30-40 population doublings, without losing important cellular functions. If the expansion procedure were to cause irreversible de-differentiation, upcyte® hepatocytes, HSC and HSEC will still be very useful for the initial development of the bioreactors to be built in WP3 and WP4. Obviously, if teams in the **SEURAT-1** cluster (e.g. *SCR&Tox*) generate cells suitable for population of liver-simulating devices, we will collaborate with them to include the most optimal cells in the ***HeMiBio*** bioreactor.

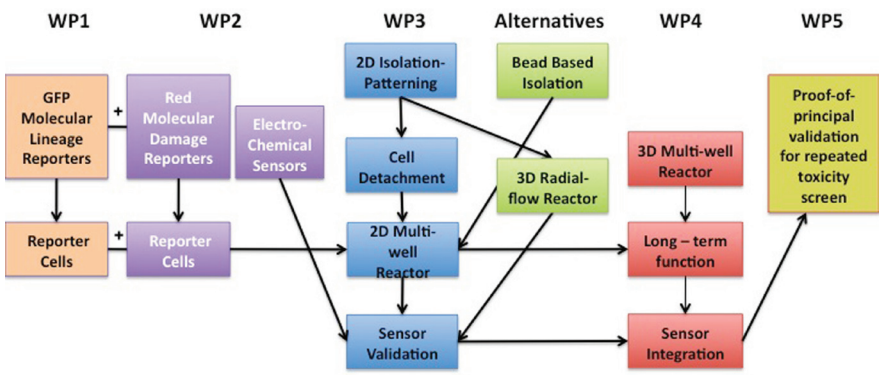

Figure 4.9 *Tool development and incorporation in diverse bioreactor designs.*

iPS cells will undergo zinc-finger-mediated homologous recombination (ZFN-HR; *Figure 4.10*) to insert (i) selection cassettes (fluorophore and/or truncated cell surface receptor) downstream of either a gene expressed specifically in mature hepatocytes, HSC or HSEC (WP1); (ii) or cell damage-specific expression cassettes (NF-βκ and caspase-3; to be determined together with DETECTIVE and ToxBank) (WP2). This combination will allow precise detection of toxic effects on any of the three cell components to be incorporated in the device. As a second means of following the cell health, we will create several electronic microsensors to be located in the direct vicinity of the cells to measure in real-time or at specific interrogation times (e.g. after a toxic insult) relevant parameters of the health of the cells (WP2). Microsensors will also be generated that can monitor cell culture conditions to aid in optimisation of the culture medium (WP2).

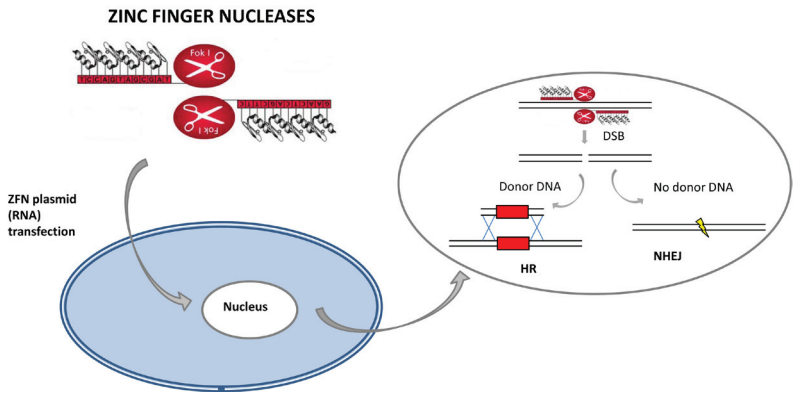

Figure 4.10 *Genetic engineering using ZFN/TALeN-HR for cell tracking / toxicity measurement.*

In WP3 and 4, sequentially more complex bioreactors will be built to culture hepatocytes *(Figure 4.10)*, HSC and HSEC 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. In WP3, we will test whether (immature) hepatocytes, HSEC and HSC can be captured from mixed iPS cell cultures by microfluidic isolation on hepatocyte, HSEC 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 (WP4).

The function of the different electronic microsensors from WP2 and the molecular sensors introduced in the cells in WP1 and WP2, will be tested first in the initial bioreactors used in WP3 and, if validated, subsequently incorporated in the final bioreactors in WP4. In WP3 and WP4 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 animal 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 in WP5 with cosmetic ingredients with suspected hepatotoxicity. A prerequisite for accomplishing this critical task is the establishment of a set of function and toxicity screening assays as well as a list of test compounds, which will be done in close collaboration with investigators of ToxBank.

Throughout WP1, 3, 4 and 5, we will use classical "-omics" and functional studies to assure that the cellular components are liver-like, and to assess the effect of the toxic compounds on that state. Different cell components will be reselected from the device using the integrated truncated cell surface receptors, as well as the transcriptome and epigenome. This will also be done in collaboration with DETECTIVE.

In WP6 (Training and Education), we plan a series of education and training opportunities to train young scientists within ***HeMiBio*** and beyond, in the highly innovative and technically challenging concepts embedded in the proposal. Finally, we employ a professional management group to ensure the smooth and professional administration and implementation of the above strategy described in WP7 (Management and Coordination).

4.3.5 Innovation

The expected improvements in generating a valuable human *in vitro* alternative for medium / long-term toxicity testing through ***HeMiBio*** as compared with the current stat of the art is given in *Table 4.2*.

Table 4.2 *Comparison of the current state of the art regarding the development of in vitro test systems and the HeMiBio approach.*

Current state of the art/problem	Innovation by *HeMiBio*
1. Although multiple projects have been funded by the EC aimed at decreasing the need for animals in toxicity testing, the assessment of toxic effects of **chronic exposure** still requires a relatively high consumption of animals. Hence, there is a need to develop *in vitro* alternatives that specifically address chronic exposure.	The aim of ***HeMiBio*** to maintain functional human hepatocytes, the chief source of CYP450 isoenzymes and UDP-glucuronosyl transferases and esterases, for more than a month in culture, would allow better characterisation of the biotransformation of drugs, and hence decrease the probability that a drug known to be active in rodent models is not active or toxic in humans. Moreover, by using iPS cells as a source of cellular components in the ***HeMiBio*** bioreactor, it would be possible to take into account the problem of variability in metabolism among humans by using cells derived from individuals with a different metabolisation profile. This will offer a valuable human *in vitro* testing alternative to assess drug toxicity earty in the drug development process and will curtail the exorbitant costs related to drug failure late during its development.
2. CYP450 enzymes are most commonly involved in drug metabolism, with 5 out of 57 human CYP450s accounting for 90% of the metabolism, while other major contributors are UDP-glucuronosyl transferases and esterases. These two enzyme systems are therefore the most important in predicting bioavailability and drug toxicity and there **activity needs to be optimally preserved** in *in vitro* testing systems.	
3. Although, these enzyme systems are present in all species, an inherent problem in toxicology is that experiments must be done in animal models and extrapolated to humans. **In humans the activity and availability of the different enzyme systems involved in drug metabolism vary significantly**, and depending on genetic variability, some people are extensive metabolisers, whereas others are poor metabolisers.	

Clinical application 1: Liver-simulating device to evaluate HBV/HCV infection mechanisms and development of antivirals

Chronic hepatitis C virus (HCV) infection is the leading cause of liver failure requiring liver transplantation. An estimated 400 million people live with chronic hepatitis B virus (HBV) infection worldwide. Each year, > 750,000 people die from cirrhosis or hepatocellular carcinoma (HCC) caused by HBV. Despite the high incidence of these liver diseases, development of drugs for HBV and HCV infections has been hampered by the lack of cell/animal models wherein the diseases can be recreated, due to the very narrow tropism of the viruses (*Gottwein, 2008*). Human hepatocytes are the predominant target of HBV and HCV infections. ***HeMiBio*** may alleviate the problem of paucity of human hepatocytes to use *in vitro* to evaluate the infection process, the influence of hepatitis viruses on cell function, and identify additional targets for drug development. Moreover, ***HeMiBio*** will generate a liver-simulating device wherein the complex architecture and cellular composition of the liver is recreated, that will enable evaluation of HBV/HCV entry via the hepatic sinusoidal endothelial cells (*Lai et al., 2006*) into the liver parenchyma.

Clinical application 2: Therapies for patients with liver failure

Yearly, 60,000 European citizens die due to liver failure, and at least ten times more are chronically affected and disabled by liver disorders. The only curative therapy for acute chronic liver failures is liver transplantation. An alternative is hepatocyte transplantation. However, also for this therapy, organ donor shortage is an impediment. A temporary therapy is to treat patients with a bioartificial liver (BAL) device to bridge the time to transplantation. Although such bioartificial livers have been tested, shortage of hepatocytes is also a major problem to further develop such devices. One of the chief goals of ***HeMiBio*** is to develop hepatocytes from human pluripotent stem cells [embryonic stem cells (ES cells) and induced pluripotent stem cell (iPS cells)]. Availability of human hepatocytes would alleviate the shortage of hepatocytes needed to perform hepatocyte transplantations in individuals with end stage liver disease or genetic forms of liver disease, either as a permanent solution for liver failure, or as a bridge to liver transplantation. Availability of human hepatocytes will also allow further testing of bioartificial liver (BAL) devices to bridge patients until liver regeneration occurs in fulminant liver failure, or until a donor organ is available (*Pryor & Vacanti, 2008; McKenzie et al., 2008*).

Bioreactors as other organ-simulating devices

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. As for the liver, the functional, morphological and molecular characteristics of the cells that constitute these organs are determined by environmental factors (e.g. the vicinity to and direct contact with other cell types in the organ, the exposure to flow and certain oxygen levels, etc.). All these parameters can be integrated in a bioreactor system, like the one we propose here for the liver. The technology developed in ***HeMiBio***, i.e. cells that are manipulated as such that their differentiation state, functionality and viability can be monitored and the inclusion of sensors that can monitor the environment of the cells, can be translated to other organ systems for high-throughput screening for the effect of drug candidates without needing animals.

An interesting example where 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. Several methods have been developed to isolate glomeruli (the filtration units of the kidney) and culture the three types of glomerular cells. For instance, concept of a nephron-on-a-chip using a MEMS-based (MicroElectroMechanical System) bioartificial device has been proposed but attempts to populate this device with the various renal cell types that constitute a kidney have not been reported (*Weinberg et al., 2008*). However, the methods suffer from impure cell populations and the short 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-dimensional glomerular organoid (*Wang & Takezawa, 2005*). The population of 2D- and 3D-bioreactors with hiPS cells 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.

Innovation in microfluidics

Microfluidics, the manipulation of fluids in channels with dimensions similar to living cells has emerged as a distinct new field (*Whitesides, 2006*). Even though it already strongly influences topics, such as chemical synthesis and biological analysis, the field is still at an early stage of development with regard to commercialisation of microfluidic products. Nevertheless, a few highly successful products such as Affymetrix's "GeneChip", Agilent's "2100 Bioanalyzer" or Febit's "Geniom" biochips confirm the impact that microfluidics has acquired in the past few years on the health sector, in particular in genomics, proteomics, cell-based assays and clinical diagnostics. Its main advantages are well known and comprise the automation of high-throughput assays, a need for low volumes of costly or rare samples, short reaction and analysis times, as well as high reproducibility, etc.

The internal spatial structure of the liver features its formidable complexity for good reasons. The ability of the liver to receive specific signals, e.g. from toxins, and to deliver information, e.g. by secreting bilirubin, occurs through a vast and dense network of microchannels. The transport of these substances takes place via two extremely important transport mechanisms: convection and diffusion or often a combination of both. On one hand, convection that mainly takes place in the blood vessels, guarantees the distribution of nutrients and oxygen, to the liver cells. On the other hand, diffusion that is extremely relevant for length scales smaller than tens of micrometres, allows the creation of gradients in the hepatic tissue. For these reasons, microfluidics is utterly indispensable when tackling the task of devising a liver-like functionality outside a living organism. ***HeMiBio*** comes up with a range of novel 2D and 3D microfluidic elements that provide a precise control of this molecular transport. It is achieved by means of innovative materials, a sophisticated combination of processing tools and a concomitant theoretical evaluation of the occurring transport phenomena.

Innovations in biosensors

"*The demand for biosensors for multiple applications is on the rise. Biosensors are proving to be effective in a wide range of medical applications. The need for efficient, compatible and user-friendly biosensors is imperative*." This quote from a recent Frost & Sullivan (2010) study (N211-32) sums up the wide industry interest in novel biosensors. The advantage of

HeMiBio's approach is that it makes use of pre-existing technologies, which are already well established at the partner's institutions. A second benefit is the portability of sensors from one target substance to another. This, of course, has its limitations, but a given method, like amperometric, i. e. redox-based sensing, is equally adaptable to measuring e. g. enzymes like lactate dehydrogenase, metabolites such as urea and glucose and inorganic solutes like oxygen. Hence, we judge the economic impact of the biosensors developed in ***HeMiBio*** very high.

The project ***HeMiBio*** will yield novel biosensors for diverse biochemical entities ranging from inorganic ions to enzymes. The cost-effective, miniaturised and highly integrated processing of these tools enables setting up automated and controlled bioreactors as required here for stably maintaining a liver-simulating device over periods of several weeks. Beyond the direct use of the sensors modules in the liver bioreactor, the future commercial use of the developed sensors will be assessed as well as their adaptability to other applications fields.

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

Jürgen Hescheler

4.4.1 Introduction

As one of the building blocks of the **SEURAT-1** Research Initiative, **DETECTIVE** focuses on a key element on which *in vitro* toxicity testing relies: the development of robust and reliable, sensitive and specific biomarkers.

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

Upon combination and subsequent integration of the various readouts, biomarkers for prediction of human long-term toxicity *in vitro* can be obtained. Based on integrative statistical analysis, systematic verification and correlation with *in vivo* data, relevant, specific, sensitive and predictive biomarkers will be selected.

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

4.4.2 Objectives

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 chronic toxicants relevant for humans.

Specifically, the **DETECTIVE** project will develop biomarkers of human toxicity in human-based *in vitro* systems by:

- Interfacing with the other building blocks of the **SEURAT-1** Research Initiative, in particular ToxBank, to substantiate knowledge and toxicological data about already existing biomarkers for chronic organ damage such as cardiomyopathies, arrhythmias, liver cirrhosis, steatosis, cholestasis, apoptosis, etc. and relevant biological processes.
- Assessing, in collaboration with ToxBank, 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, kidney and ES cells derived somatic cells but 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 and heart as well as for ES cells derived somatic cells, but possibly also for other model systems as provided by other building blocks. 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 that allows 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.
- Integrating functional with "-omics" readouts in *in vitro* model systems by taking into account the reversibility of effects. Defining thresholds of concern for chemical stressors that define escalating severities of biological responses.
- Addressing qualification of biomarkers (the evidentiary process linking the biomarker to a clinical observation), aspects of sensitivity, specificity and reproducibility of the various readouts and other requirements for regulatory acceptance of biomarkers.

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

➠ Formulating GLP-compliant SOPs on procedures leading to the identification of the most robust and predictive biomarkers.

4.4.3 State of the Art

Currently, no available alternatives to animal testing for detecting toxicity after repeated exposure (e.g. equivalent to 28- or 90-day *in vivo* studies, according to OECD TG407/408) are accepted for regulatory purposes. A 2002 ECVAM Working Group on Chemicals report pointed out that "*the readiness of in vitro models for long-term effects to undergo pre-validation and validation will depend on progress made at the research and test development level*" (*Prieto et al., 2006*).

Classical *in vitro* cytotoxicity tests are not suitable for the detection of repeated dose effects since they focus on late events during cell death mainly associated with necrotic or apoptotic processes (*Vinken et el., 2009*). As significant sub-lethal effects related to low-dose exposure cannot be detected by conventional cytotoxicity tests, new technologies allowing the detection of side effects occurring in an early phase of toxicity need to be applied, such as *high content screening platforms* or the employment of fluorescence-based reagents, quantitatively analysing cellular targets and physiological processes. Most *in vitro* assays for toxicology provide a dose-response relationship for a particular combination of test substance and cellular system. Such dose-response-relationships, however, cannot assess the dynamic aspects of the cellular response to a toxin, or the recovery of exposed cells after withdrawal of the test substance (reversibility). So far most toxicity screening approaches involve non-discriminative toxicity endpoint measurements (i.e. cell death, MTT assay), which do not integrate chemical class-specific effects. Yet, different chemicals affect various essential cellular processes at the sub-cellular compartment level. Indeed, analyses of toxicogenomics datasets indicate that compounds affect a variety of cellular biological processes. So far, these different processes can *not* be determined by a unified toxic endpoint. Recent advances in automated fluorescence microscopy, quantitative multi-parameter image analysis and data mining in combination with BAC recombineering now allow efficient GFP tagging of a gene in its own genomic context, allowing physiological expression of the tagged protein.

Epigenetic alterations are potentially more damaging than nucleotide mutations because their effects on regional chromatin structure can spread, thus affecting multiple genetic *loci*. They also tend to affect a high proportion of those exposed, unlike conventional mutations, which are relatively rare (*Jirtle and Skinner, 2007*). The most common changes are alterations in the methylation pattern of DNA, but modifications of histone proteins are also implicated (*Hirst and Marra, 2009*). Clear evidence shows that epigenetic inheritance of disease

stays for generations after the initial exposure, especially in two cases involving endocrine disruptors (*Pietro et al., 2006; Hartung et al., 2008*). In this context, it is of major relevance to study epigenomic alterations as a mechanism underlying repeated dose (chronic) toxicity. It is of particular interest to test the hypothesis that epigenomic changes induced by model compounds for repeated dose toxicity persist in *in vitro* models upon ending exposure.

DNA methylation is one of the main epigenetic regulatory mechanisms that are generally associated with the initiation and maintenance of silencing of gene expressions. Aberrant DNA methylation can occur as hypo- or hypermethylation and either form can lead to chromosomal instability and transcriptional gene silencing (*Klose and Bird, 2006*). There is a growing body of evidence that chemical exposures can induce changes in DNA methylation patterns, indicating that a full understanding of this type of epigenetic change is required to get insight into molecular mechanisms of action (*Rusiecki et al., 2008*). As such, DNA methylation analysis may contribute to biomarker development for the prediction of chemical toxicity, particularly in combination with gene expression analysis and other markers of epigenetic changes.

The post-translational modification of histones through acetylation, methylation, phosphorylation and other processes serves to regulate the structure of chromatin and determine how the DNA sequence is interpreted. Together with DNA methylation they are often referred to as the epigenome. Genome-wide disruption of histone acetylation in response to an environmental carcinogen has been demonstrated (*National Research Council of the National Academies, 2007*). Changes in the 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. For instance, suppressing histone deacetylation with specific inhibitors is known to stabilise the liver-specific nature of primary hepatocytes (*De Kock et al., 2009; Ceelen et al., 2011*). Thus, stabilisation of histone acetylation patterns or alteration by external stimuli, can be interpreted as major responses to chemical exposures, and may be involved in gene expression mediated toxic responses.

MicroRNAs (miRNAs) have emerged as powerful negative regulators of mRNA levels in several systems (*Hudak and Novak, 2008*). These miRNAs can be held responsible for influencing mRNA levels of important genes involved in metabolic and toxicological pathways. Increasing evidence has implicated miRNAs in biological processes such as normal development and disease pathology, particularly in cancer (*Farazi et al., 2011*). The fact that miRNA transcription involves polymerase II promoters, which frequently contain toxicologically significant enhancer regions, implies that miRNAs are also expected to play an important role in xenobiotic responses in cells. For instance, the peroxisome proliferator-activated receptor alpha (PPARα) agonist Wy-14,643 down-regulates the expression of let-7C, which in turn reduces let-7C-mediated repression of c-myc translation. This increases c-myc-induced expression of the proto-oncogenic miR-17-92 cluster, resulting in hepatocyte proliferation (*Shah et al., 2007*).

Proteomics are a means to assess early/immediate biological responses such as e.g. phosphorylation and oxidation of proteins, which are not detectable by transcriptomics technologies. These posttranslational modifications have a direct impact on enzyme activities and protein-protein interactions. Only at downstream stages gene transcription is activated. Usually the first response after stimulation of cells can be seen in phosphorylation of heat shock proteins and other components of stress responses (*Groebe et al., 2010*).

The systematic analysis of metabolism (metabolic profiling, metabonomics or 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 and Athersuch, 2007*).

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 and also the methodologies by which it can be assessed.

In a recent report issued by the European Commission who invited stakeholder bodies (industry, nongovernmental organisations, EU Member States, and the Commission's Scientific Committee on Consumer Safety), selected experts assessed the status and prospects of alternative methods and provided a scientifically sound estimate of the time necessary to achieve full replacement of animal testing (*Adler et al., 2011*). In summary, the experts confirmed that it will take at least another 7–9 years for the replacement of the current *in vivo* animal tests used for the safety assessment of cosmetic ingredients for skin sensitisation. However, the experts were also of the opinion that alternative methods maybe able to give hazard information, i.e. to differentiate between sensitisers and non-sensitisers, ahead of 2017. This would not provide the complete picture of what is a safe exposure because the relative potency of a sensitiser would not be known. For toxicokinetics, the timeframe was 5–7 years to develop the models still lacking to predict lung absorption and renal/biliary excretion, and even longer to integrate the methods to fully replace the animal toxicokinetic models. For the systemic toxicological endpoints of repeated dose toxicity, carcinogenicity and reproductive toxicity, the time horizon for full replacement could not be estimated.

"Systems toxicology" has been proposed as an innovative new discipline to make a major step forward in the development of alternative human safety testing. *Figure 4.11* shows how different technologies interact and could eventually lead to the development of a "systems toxicology", by combining *"in essence, various new, information-rich technologies […] with*

established scientific knowledge (knowledge of biochemical pathways, knowledge of patterns/ toxicity signatures; knowledge of biomarkers; knowledge of pharmacokinetic and chemical properties) using computational approaches" (*Hartung et al., 2008*).

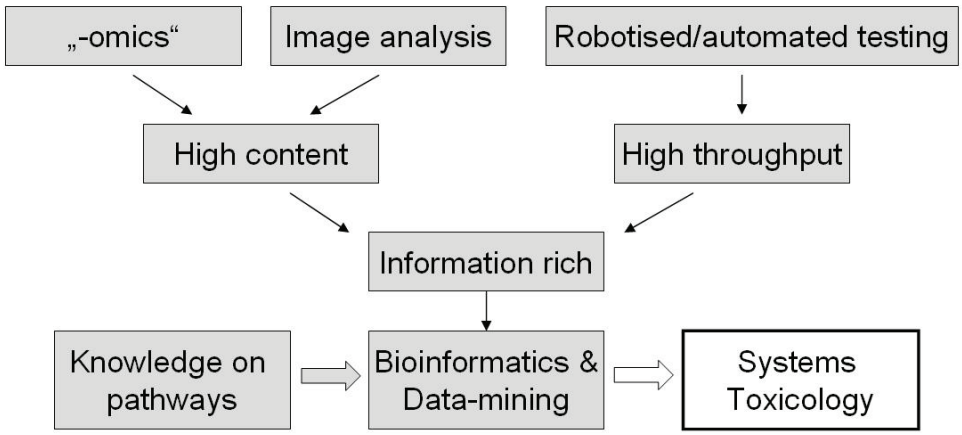

Figure 4.11 *Towards systems toxicology (adapted from Hartung et al. 2008).*

The **DETECTIVE** project, combining a battery of established and innovative high content and high throughput functional and "-omics" technologies to establish new biomarkers for repeated dose toxicity, is such an approach. The topics coloured in light grey in *Figure 4.11* highlight the different elements of the "systems toxicology" approach that are covered by **DETECTIVE**. With regard to this approach, the fruitful integration with the other building blocks of the **SEURAT-1** Research Initiative, in particular COSMOS ("Computational modelling and estimation techniques"), NOTOX ("Systems biology for predictive models") and ToxBank ("Integrated data analysis and servicing"), will further strengthen the impact of the proposed work.

4.4.4 Approach

The **DETECTIVE** project will develop candidate biomarkers relevant for repeated dose toxicity, using cellular *in vitro* models of primarily human tissues (derived from primary cells, stem cell-derived somatic cells or cell lines), including hepatocytes, cardiomyocytes and renal epithelial cells or other cells of toxicological relevance, and well-defined, relevant test substances as provided by ToxBank. For the establishment of these biomarkers, **DETECTIVE** applies an innovative approach combining high performance functional readouts with state of the art "-omics" technologies. While functional parameters will provide insights into the physiological effects of toxicants on specific cell functions, "-omics" techniques will deliver data on the

entire cellular situation at the molecular level. With regard to long-term, repeated dose toxicity testing, it is of particular importance to be able to detect early toxicity markers further to low-dose exposure, which does not directly result in acute toxicity. By applying sensitive "-omics" technologies to assess chemically induced changes in cellular biochemistry and correlating this information to sensitive endpoints relevant to cellular function (i.e. targeted endpoints with known importance), and *vice versa*, **DETECTIVE** will be able to identify key events of adverse effect outcome pathways, which can be further assessed for their suitability as biomarkers relevant for repeated dose toxicity. This concept is illustrated in *Figure 4.12*.

The potential of such a combination of "-omics" technologies with organotypic *in vitro* models, promising to achieve further mechanistic insights into cellular responses to chemical insults, which may be highly sensitive and specific, has been underlined at the ECVAM Workshop 56 (*Pietro et al., 2006*).

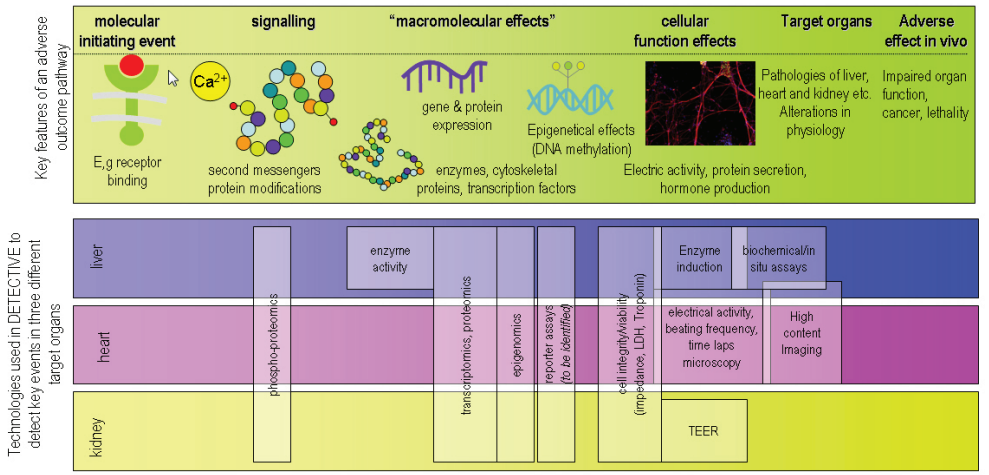

Figure 4.12 *The conceptual framework describes a sequence of effects in ascending order which starts with a molecular initiating event in which a substance interacts with a biological target molecule. This event will trigger a sequence of follow-up events, which will ultimately result in an adverse effect in vivo. These reactions at different levels are part of an adverse outcome pathway (AOP). In order to get an insight view on the different events,* ***DETECTIVE*** *brought together the full range of emerging technologies allowing an insight into the mechanistic understanding of the pathways, which will allow the identification of the most predictive biomarkers.*

Figure 4.13 below shows how such elucidation of changes at the molecular level and corresponding toxicity pathways, correlated to biological function, can for example help distinguish adaptive stress responses from toxic effects leading to cell damage.

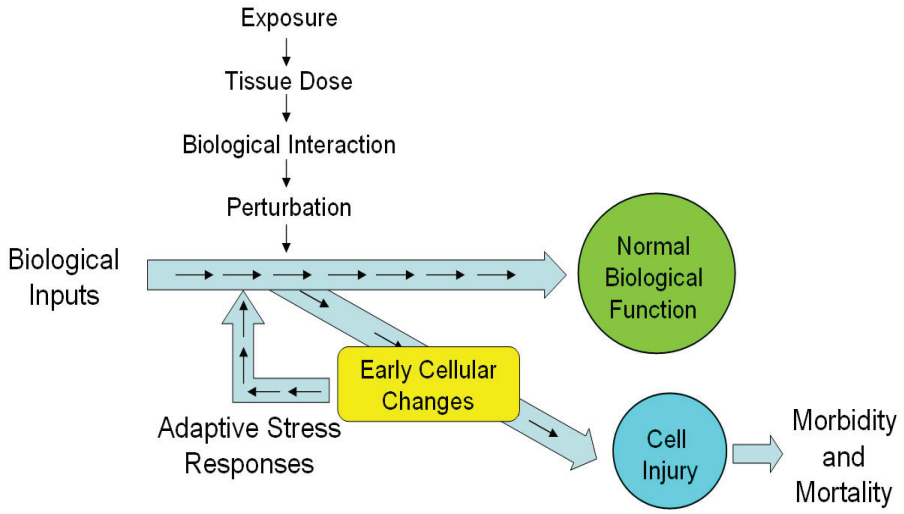

Figure 4.13 *Proposed paradigm shift in toxicity testing: activation of toxicity pathways (taken from: National Research Council of the National Academies, 2007).*

The **DETECTIVE** project, as part of the integrated strategy of the **SEURAT-1** Research Initiative, will build on the possibilities offered by such a combinatory approach, which allows working with increasing complexities from genomics to functional readouts. The partners have carefully selected the technologies to be used in the project, based on criteria such as applicability to different cell systems, robustness, proven reliability in similar settings such as investigative toxicology, balance between technologies suited to deliver qualitative or quantitative results, cost efficiency and automatability in view of later industrial use. The technologies chosen include both high content technologies, which have the capacity for multi-parameter measurements, and/or high throughput screening platforms, which will be used to reliably identify potential markers by exposure to large chemical libraries. Thus, **DETECTIVE** comprises the required state-of-the-art tools and expertise, generated in previous research projects, to move beyond descriptive science and towards mechanism-based prediction.

In a first step, existing cellular models of human heart, liver and kidney will be examined for their applicability to long-term and repeated dose toxicity testing. Selection of the *in vitro* test systems will be based on ECVAM's criteria for entering into pre-validation (*Balls and Fentem, 1999*) and GCCP principles will apply. If available, well-known clinical markers of chronic organ damage will be defined for use as reference markers in the initial set of *in vitro* screening systems. These cell systems will then be exposed to test substances as defined by ToxBank and identified in other projects.

After having developed exposure protocols suitable for repeated dose toxicity testing,

biomarkers that occur early in the process of long-term toxic responses and that are mechanistically linked to underlying pathology will be identified by using a panel of functional and "-omics" technologies. Different treatment schemes will be compared in order to mimic the repeated dose effect. These will include protocols for continuous and discontinuous treatment, or longer exposure periods. Also subject to investigation may be scenarios that permit assessing reversibility of effects after withdrawal of substances. Obtained dose-response curves will give a first insight into the stability, accumulation and low dose effects in a cellular system. The models will be treated with a set of well-known compounds in order to obtain initial feedback on the sensitivity and specificity of the readouts.

The project will focus on the development and qualification of robust biomarkers for the affected target organs: liver, heart and kidney. However, the strategy of combining functional and "-omics"-readouts developed in the project for establishment of biomarkers will also be applicable to other organ models currently not covered by the project, such as the nervous system, which might be provided by *SCR&Tox.* Furthermore, some biomarkers, for example markers of oxidative stress may be measured in a specific organ, but may be applicable to other organs as well.

Based on integrative data analysis of the data generated by the different readouts and on correlation with existing *in vivo* data, the most relevant, highly predictive repeated dose biomarkers will be selected in a pathway- and evidence-based approach. Requirements of regulatory acceptance of biomarkers, their qualification (including the evidentiary process linking the biomarker to a clinical observation), aspects of sensitivity, specificity and reproducibility of the various techniques will specifically be addressed in the selection and verification process of the developed biomarkers.

DETECTIVE will thus establish a solid and reliable basis on which a future *in vitro* test system used by industry can be built on. The work has been divided in four sub-projects (SPs), each composed of several work packages (WPs), as summarised in the following *Table 4.3*. A detailed description about the specific tasks and approaches is given below.

Table 4.3 *Overview about the* ***DETECTIVE*** *project structure.*

Sub-projects	Central	Functional readouts	„-omics" readouts	Integration of biomarkers
Work packages	Strategy and coordination	Electric activity	Gene expression profiling	Bioinformatics and statistics
	Cell systems	Impedance measurements	Epigenetics and miRNA profiling	Verification, stabilisation and selection of biomarkers
	Management	Imaging (HT)	Proteomics	
	Training	Cell type specific readouts	Metabonomics	

Sub-project 1: Central work packages

DETECTIVE has defined a set of central work packages that address transversal topics in the project. These are grouped in sub-project 1.

At project start, **DETECTIVE** will organise a launch meeting together with other building blocks, in order to coordinate the research, identify any possible overlaps and agree on an overall time table for inputs and outputs in the different building blocks. This will be carried out in work package 1. This work package also includes the overall scientific coordination and strategy.

The **DETECTIVE** consortium will start by using available cell systems as provided by project partners and later the optimised systems developed by *SCR&Tox* and *HeMiBio*. Quality control and verification of the applicability to readout technologies, stability and reproducibility of all cell systems will be carried out in work package 2. This will include performance tests with the cells across the partner laboratories using the cells for specific readouts. As harmonised exposure protocols are key requirements to identify changes at cellular level that are indicative for long-term toxicity following repeated dose administration, various exposure protocols will be compared to select the most appropriate treatment scheme. These protocols will be used consistently throughout the project. The relevant exposure protocols for long-term toxicity should support the determination of biologically effective doses relevant for COSMOS.

Three work packages will address project management (work package 3), training (work package 4) and dissemination and exploitation (work package 5).

Sub-project 2: Functional readouts

The overall objective of sub-project 2 is to develop functional readouts for the identification of biomarkers of repeated dose toxicity for multiple target organs *in vitro*.

The specific objectives of sub-project 2 are:

- To complement toxicogenomics (transcript, protein and metabolite profiling) derived by sub-project 3 with cellular responses.
- To develop a panel of cytomic assays.
- To identify new potential biomarkers relevant for hepatotoxicity, cardiotoxicity and nephrotoxicity or for other cell types of toxicological relevance.
- To perform cell type specific readouts, such as albumin or urea secretion, phase I and II metabolising activities of hepatocytes, or protein release from cardiomyocytes. To test which of the conventional functional readouts are essential and which can be replaced by faster techniques without losing predictive power.

Scientific information derived from "-omics" technologies in sub-project 3 will be complemented by functional readout systems optimised in sub-project 2 allowing insights into cellular responses to chemical insults. In particular, toxic effects on excitable cells such as cardiomyocytes as well as on the functionality of hepatocytes will be analysed in detail in order to understand mechanistic aspects of these target organ toxicities at cellular level. Vice versa, endpoints identified in the functional readouts will be submitted for "-omics" analysis to gain a more comprehensive mechanistic understanding of the observed effects. A panel of cell-based assays (cytomics) that enables linking genomics, proteomics and metabonomics readouts with the dynamics of cellular functions will be established.

High-content image analysis as well as impedance measurements provide the unique possibility for continuous monitoring of major cellular aspects, such as migration, proliferation, cell morphology, cell-cell interactions, and colony formation. These functional measurements are thus very adequate to repeated dose experiments and will provide an assessment of the effects of repeated dose or long-term application of compounds. 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. These continuous readout systems will be accompanied by established technologies such as measurement of electric activity using multielectrode arrays (MEA) or cell type specific readouts.

The information thus gained by sub-project 2 will allow further development of relevant intermediate biomarkers deriving from sub-project 3 into surrogate endpoints that can be used for the prediction of long-term toxicities (sub-project 4). An understanding whether observed effects are due to non-specific or specific cellular failures will support the qualification of biomarkers by sub-project 4. This will increase the confidence in the predictive value of the biomarkers for assessing the risks of toxicity. The dose-response curves obtained in sub-project 2 will provide a better understanding of thresholds of concern leading to functional failures of various cell types.

Hereafter, the technologies used for the functional readouts, and how they can lead to the development of novel toxicity biomarkers, are described in more detail.

Electrical activity: The contractile properties as well as the electrophysiological characteristics of electrically active cells such as cardiomyocytes can be monitored in real-time using multielectrode arrays (MEA; *Figure 4.14* and *Figure 4.15*). Activation, repolarisation and conduction properties of stem cell-derived cardiomyocytes after exposure to test substances will be assessed by measuring extracellular electrograms in repeated dose scenarios. The MEA technology will thus allow qualification of candidate biomarkers of e.g. cardiotoxicity by assessing the cellular physiology. Contraction of the cardiomyocytes will also be recorded using the cell sensor impedance technology (see below) at the same time as the MEA recordings, allowing direct comparisons to determine whether the electric potentials correlate

with the magnitudes of the contraction. Differences between both profiles may allow additional insights in the functional coupling of both events in cells exposed to test substances. This approach will be applicable also to other excitable cells such as neurons.

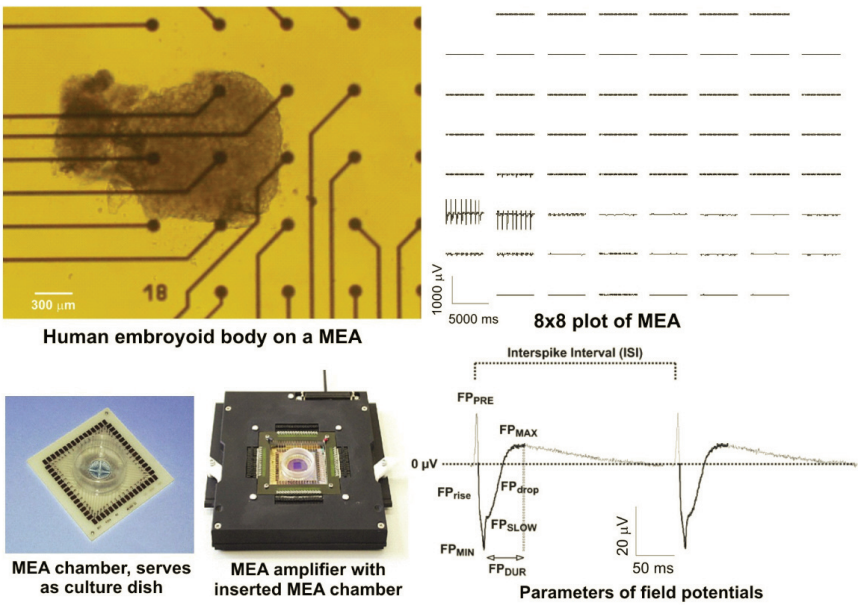

Figure 4.14 *Human ES cell-derived cardiac bodies plated on MEAs used for QT screening (taken from: Liang et al., 2010).*

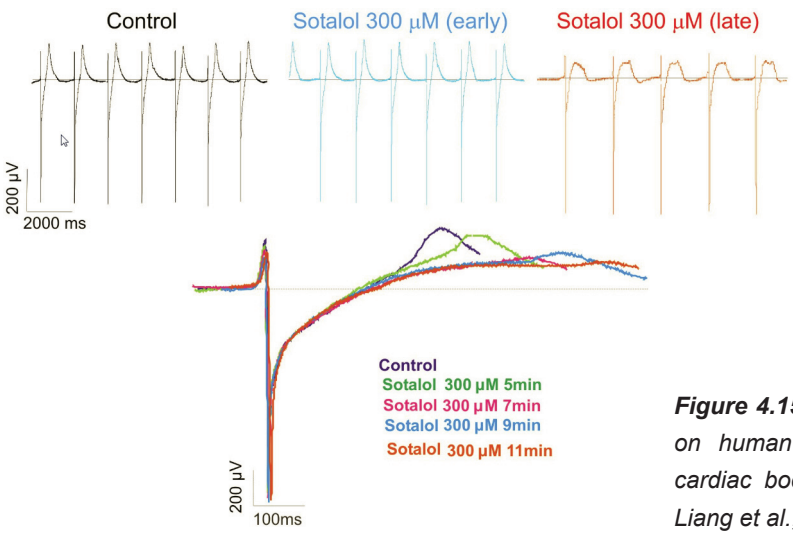

Figure 4.15 *Effect of Sotalol on human ES cell-derived cardiac bodies (taken from: Liang et al., 2010).*

Impedance measurements: Impedance measurements allow continuous, multi-parametric monitoring of cell function and integrity in situ. The consortium will apply the xCELLigence impedance measurement system provided by **DETECTIVE** partner Roche (*Figure 4.16*).

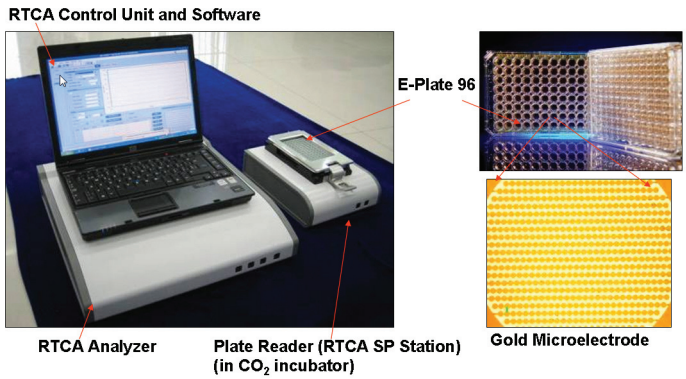

Figure 4.16 *xCELLigence system components (© Roche Applied Sciences).*

The xCELLigence instrument utilises an electronic readout by impedance measurement to non-invasively quantify adherent cell proliferation and viability without requiring additional labelling. The cells are seeded in micro-titer plates containing microelectronic sensor arrays, so-called E-plates. The interaction of cells with the gold electrodes covering the well bottom generates a sensitive cell-electrode impedance response that not only detects cell attachment, density and growth, but also morphological changes and even cell function in real-time (the principle is shown in *Figure 4.17*). Since E-plates are kept in common cell culture incubators throughout the measurements, cells can be monitored continuously for many weeks. This enables long-term recording during repeated dose compound treatments.

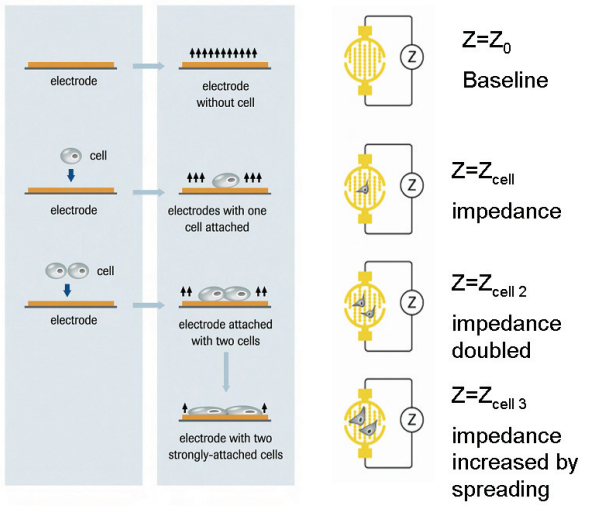

Figure 4.17 *Principle of impedance measurement (© Roche Applied Sciences).*

An extension to monitor cardiomyocyte contraction is currently in development at Roche. This “Cardio system” is also based on impedance readings but unlike the current systems, measures 70 times per second. For the first time, this offers the opportunity to record compound-induced beating variability in a high throughput 96 well format. The currently used MEA systems are not available as high throughput versions due to the complexity of the electrode setup. In addition, MEAs can only measure compounds causing changes in the electric potential, but not in those that interfere with the contraction of the cells. In this context, cardiac arrhythmias can be induced by drugs or other compounds. Over 100 drugs were identified as suspected of being arrhythmogenic, including antimicrobial (macrolides), antifungal, antimalarial/antiprotozoal, antihistamines, gastrointestinal, psycho-tropic (tricyclic antidepressants & serotonin re-uptake), antipsychotic, diuretics and antimigraine compounds (*Schutte & Obel, 2002*). Amongst cosmetics, ingredients such as Bis(2-ethylhexyl)phthalate, contained in nearly all cosmetic chemical products, cause arrhythmia (*Gillum et al., 2009*) and also excipients such as propylene glycol can lead to cardiotoxicity (*Osterberg & See, 2003*).

In summary, these systems provide a simple and efficient assay for measuring side effects of compounds on hepatocytes and cardiomyocytes.

(High throughput) imaging: Thanks to the enormous progress made in imaging techniques during recent years and the possibilities of automated image analysis, imaging technology represents an attractive alternative or an important complement to other techniques, such as the biochemical and “-omics” readout techniques.

The use of fluorescence-based reagents permits to quantitatively analyse cellular targets and physiological processes. Standard protocols for effects on cell motility, spreading, viability, cell compartments, cell membrane and nucleus translocation as well as protocols for apoptotic processes have been developed by partners and will be adapted to repeated dose applications. In addition, selected cell type-specific proteins will be stained and quantified by immunofluorescence methodologies.

For repeated dose studies in particular, it is necessary to observe cells over extended periods of time. Non-destructive methods of observation are therefore necessary. *Time-lapse microscopy* is a technology that is ideally suited to this task, enabling the automated monitoring of cell cultures for extended periods of time (e.g. over multiple days) and amenable to upscaling (96 well-plates) via the use of robotic platforms. Parameters evaluated will include dynamic morphology, cell adhesion, motility and cell death. Information about the heterogeneity of the response to the toxicants will also be obtained and recovery of exposed cells after withdrawal of the test substance, if any, will be evaluated.

A high throughput and high resolution light microscopy, live cell imaging and image analysis system will be used for the development of a screening assay for chemical class-specific

cell-biological perturbations. Partner UL (Universiteit Leiden) has already established several BAC GFP-reporter cell lines with specific cellular localization (*Figure 4.18* and *Figure 4.19*). In addition, morphometric image analysis has allowed UL to quantify compound-specific morphological changes at the cytoskeletal level. **DETECTIVE** will build on this expertise and use these novel strategies to develop specific sets of cell lines to monitor subcellular compartment perturbations. We will systematically test the suitability of these cell lines for compound safety evaluation. Furthermore, these cell systems can be combined with time-lapse microscopy which will allow us to capture the dynamic effects of exposure to toxic substances in a cell culture over an extended period of time (e.g. over multiple days).

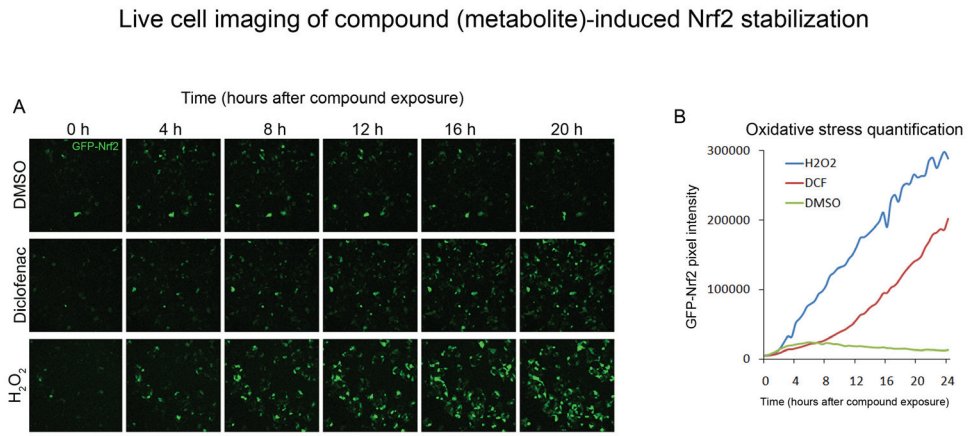

Figure 4.18 *Live cell imaging of compound (metabolite)-induced Nrf2 stabilization. A: Confocal image series of HepG2 cells stably expressing GFP-tagged Nrf2. In contrast to DMSO treatment, diclofenac and H_2O_2 treatment lead to increased intracellular levels of GFP-Nrf2 within 24 hours. B: Quantification of the Nrf2 stabilization oxidative stress assay. The GFP-Nrf2 pixel intensity is calculated as the product of the total number of fluorescent pixels above background and their intensity (© Division of Toxicology, LACDR, Leiden University, unpublished data).*

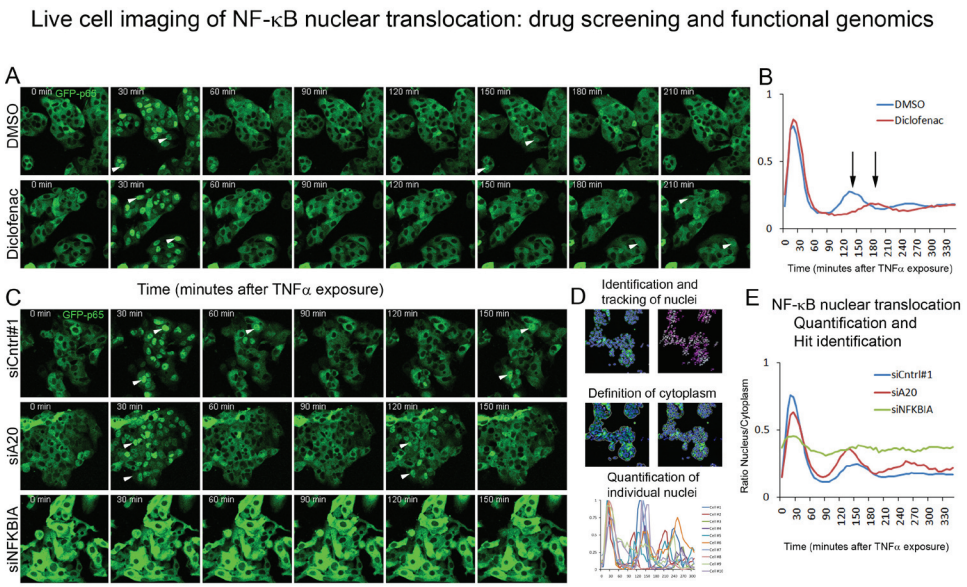

Figure 4.19 *Live cell imaging of NF-κB nuclear translocation: drug screening and functional genomics. A: Confocal image series of HepG2 cells stably expressing GFP-tagged p65 (NF-κB subunit) stimulated with 10ng/mL TNFα. Images were recorded at a frequency of 10 frames/hour directly upon addition of TNFα. Shown is the difference of especially the second nuclear translocation of GFP-p65 between control treatment (DMSO) and 8 hour drug pre-treatment (Diclofenac) (white arrowheads). B: Quantification of the GFP-p65 nuclear over cytoplasmic intensity ratio clarifies the nuclear translocation delaying effect of diclofenac on NF-κB. C: Confocal image series of HepG2 GFP-p65 cells stimulated with TNFα 72 hours after siRNA transfection. Whereas a non-targeting siRNA (siCntrl#1) induces an identical nuclear translocation profile as DMSO treatment (panel A), knockdown of A20 (TNFAIP3) leads to faster NF-κB oscillation, and the loss of IκBα (NFKBIA) totally prevents the oscillation. D: Stepwise explanation of the NF-κB quantification process. To calculate the individual cell's nuclear GFP-p65 intensities, step 1 segments the nuclei and tracks their movement from frame to frame. Step 2 is the segmentation of the cytoplasm. Step 3 calculates and normalises the GFP-p65 nuclear/cytoplasmic intensity ratio responses of individual cells. E: Average nuclear translocation graphs of siA20 and siNFKBIA clarifies their effect on NF-κB oscillation versus siCntrl#1 (© Division of Toxicology, LACDR, Leiden University, unpublished data).*

Cell type specific functional readouts: Cell type specific functional assays and classical reference assays will be used to study effects of toxicants on hepatic, renal or cardiac function. Selected relevant test substances with known toxicity selected by ToxBank will be applied to compare the predictive power of well-established functional tests to novel

biomarkers identified in **DETECTIVE**. Since some of these assays are more time-consuming and less applicable to HTS compared to reporter assays, we will investigate which of the more laborious conventional assays can be replaced by less time-consuming innovative assays without losing predictive power.

A second contribution will be cell type specific reporter assays. From previous research activities of the partners IFADO (Leibniz Research Centre for Working Environment and Human Factors at the Technical University of Dortmund) and UL (University of Leiden), a battery of diagnostic genes is available, that will be complemented and optimised in the **DETECTIVE** project. Based on the optimised list of diagnostic genes, cell type specific reporter assays will be established. These assays have to be highly cell type specific, because there is only little overlap between genes that indicate hepato-, cardio- or nephrotoxicity. Using repeated administrations in long-term experiments, the reversibility versus irreversibility of toxic effects will be examined. In the second phase of **DETECTIVE**, we will evaluate which reporter assays reliably identify certain classes of compounds and specific toxic mechanisms in order to establish a strategy how to include them into the pipeline for high throughput *in vitro* screening.

Sub-project 3: "-omics" readouts

The overall objective targeted by sub-project 3 is to improve traditional biomarkers of toxicity by gathering and integrating data on transcriptomic, proteomic, metabonomic and epigenomic responses to exposure in human *in vitro* models, which will culminate in a novel set of mechanism-based intermediate biomarkers of repeated dose toxicity applicable to evaluate the safety of different substances. Specific objectives of sub-project 3 are:

- To investigate global transcriptional responses of cells exposed to test substances (whole genome mRNA microarrays)
- To investigate epigenetic responses (DNA methylation and histone acetylation) in samples from selected *in vitro* studies using cellular models for predominantly the liver and heart and possibly other organs
- To perform whole genome miRNA analysis in samples
- To perform integrated transcriptomics – epigenetics data analysis and mRNA – miRNA data analysis and data interpretation in order to identify compound-induced modulation of toxicologically relevant molecular pathways selected *in vitro* studies using cellular models for the liver, heart and possibly other organs
- To identify and investigate biomarkers for repeated dose toxicity at the level of protein expression and posttranslational modifications
- To identify and investigate metabolic biomarkers for repeated dose organ toxicity

SP3 will develop intermediate biomarkers for repeated dose toxicity to the liver and to the heart, but also kidney and other organs, by taking the multiplexed “-omics” approach.

Well-selected samples from case studies on cellular models for mainly liver, heart and kidney toxicity, will be subjected to whole genome analysis of epigenetics (DNA methylation and histone acetylation) in combination with transcriptomics (mRNA, miRNA), as well as to proteome and metabolome analysis. Data will be assessed for quality, normalised and uploaded to the centralised data warehouse in ToxBank and (in collaboration with sub-project 4 and ToxBank) subjected to an initial statistical analysis. Readouts from individual “-omics” platforms will be integrated – again in collaboration with sub-project 4 and ToxBank – and common denominators representing perturbed pathways will be identified. These pathways will then be associated with, and thus validated against corresponding functional readouts provided by sub-project 2. Rather than developing “-omics”-based classifiers through statistical approaches by using a very large range of chemicals, the focus here will be on unravelling molecular mechanisms of action for repeated dose liver, heart and kidney toxicity *in vitro*. The main result therefore will be a set of intermediate biomarkers for mainly liver and heart toxicity in contrast to toxic responses in general, represented by well-defined and well-validated genomic pathways. This may effectively be operationalised into a limited set of genes/ mRNAs/ miRNAs/ proteins/ metabolites, which may be transferred to a low-density screen, usable as an intermediate marker of repeated dose toxicity *in vitro*.

Results will be fed back to the central project (sub-project 1) for the purpose of designing and performing additional studies, customised to answer such important research questions as the possible reversibility of observed intermediate effects and the possible identification of dose- and time-dependent thresholds in critical effects. This will generate a second series of multiplexed “-omics” analyses and subsequent (integrative) data analysis similar to what has been described above.

Gene expression profiling: Transcriptomics analysis as a state of the art, high throughput technology will be used to identify gene expression signatures affected by test compounds in different repeated dose exposure protocols (*Figure 4.20*).

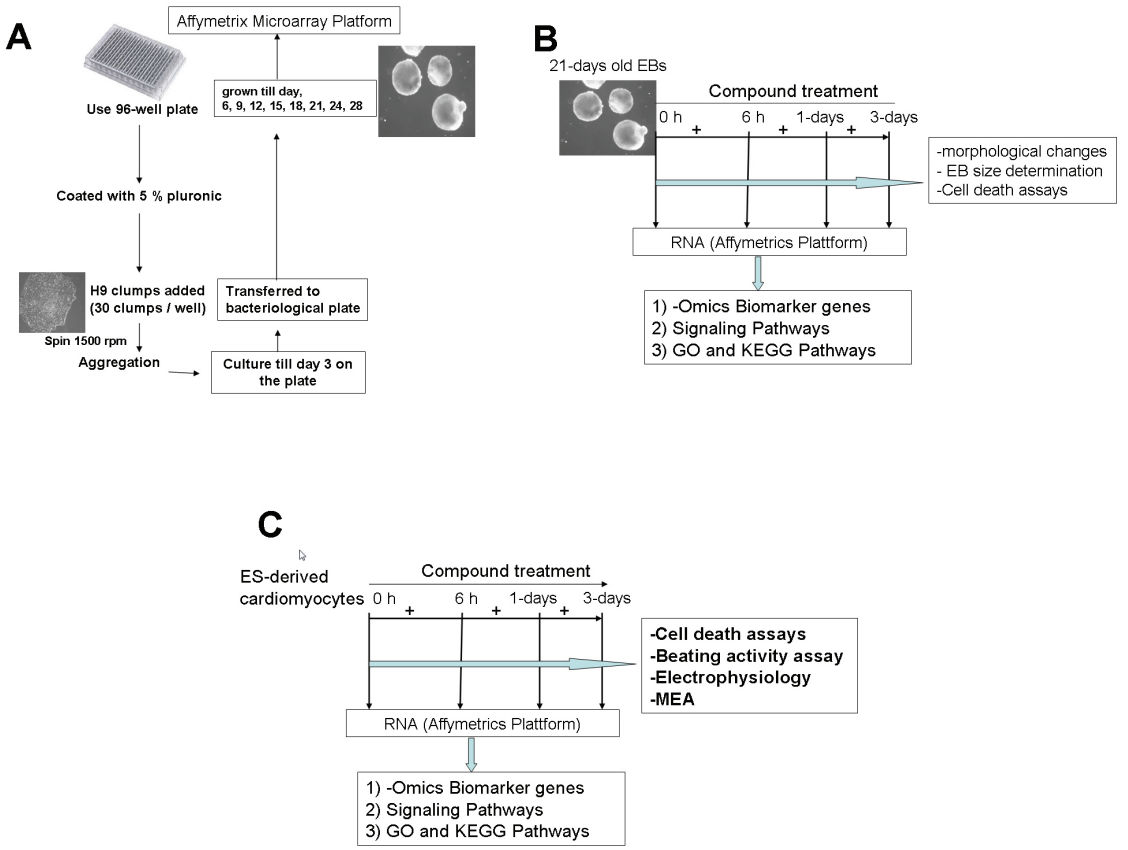

Figure 4.20 *Experiment approach scheme for repeated dose treatment of the late EBs with the different compounds (© Agapios Sachinidis, UKK).*

In the initial phase of the project, gene expression profiling will be used to investigate several fundamental questions, such as the link of genes to toxicological versus adaptive processes in order to establish a robust and consistent gene expression model for toxicological approaches based on human ES cells. The complete transcriptome of the differentiated (7-, 14-, 21-, 28-, 35-, 42-, 54- and 61-days old) EBs will be identified to determine the time point of differentiation from where on the transcriptome remains stable (*Figure 4.20A*), indicating the formation of all the different somatic cells. Then EBs with a stable transcriptome will be used for repeated dose treatment with different compounds, according to the treatment protocol as indicated in *Figure 4.20B*. In addition, cardiomyocytes derived from hES cells will be treated in a repeated dose toxicity manner with standard cardiotoxicity compounds, according to the protocol scheme as shown in *Figure 4.20C*.

In parallel, we will offer a centralised gene expression microarray service, i.e. performing '-omics' analyses for other partners within the consortium, as well as providing a data handling unit to ensure data compatibility of all the **DETECTIVE** partners. The development of a common database for management and storage of microarray data, together with uniform standards and ontologies are crucial to managing and sharing these data. Sample isolation and labelling will be performed by means of an automated set up. RNA samples will be hybridised on Affymetrix GeneChip® Array Plates. Data will be statistically analysed using ArrayTrack™ (National Center for Toxicological Research, FDA, USA) and the statistical programme R. Identified affected genes will be verified by qRT-PCR using Real Time Ready assays developed by Roche. Interesting candidate genes that are highly expressed after exposure to a compound will be additionally verified using the shRNA approach.

Epigenetics and miRNA profiling: The **DETECTIVE** project will study epigenomics at the level of DNA methylation and histone acetylation. Aberrant DNA methylation and its effects on chromosomal instability and transcriptional gene silencing upon chemical exposures indicate that insights into this type of epigenetic change could result in a better understanding of molecular mechanisms of action. The analysis of DNA methylation in combination with gene expression analysis, as well as other markers of epigenetic changes, will contribute to developing biomarkers for the prediction of chemical toxicity. The methylated DNA immunopreciptation (MeDIP) method will be used for enrichment of methylated genomic DNA. Both enriched and reference DNA will be labelled using the NimbleGen Dual-Color DNA Labelling Kit, and hybridised to the 2.1M deluxe NimbleGen promoter array in a NimbleGen Hybridisation System. These promoter arrays enable the analysis of all known CpG islands, and have an extensive coverage of all annotated gene (~ 10 kb) and miRNA promoters (730). The arrays will be washed, dried, and subsequently scanned on a NimbleGen MS 200 scanner. The generated data set will be analysed using the NimbleScan software package.

As stated above (see chapter 4.4.3), stabilisation of histone acetylation patterns or alteration by external stimuli can be interpreted as major responses to chemical exposures, and may be involved in gene expression mediated toxic responses. In order to establish such epigenetic responses, the distribution of chromosome-wide acetylated histone (focusing on Histone H3 and H4) will be determined after enrichment of DNA fragments with high levels of acetylated histones using immunoprecipitation. This will be followed by hybridisation to the same 2.1M deluxe NimbleGen promoter array as used for the DNA methylation analyses. Data are analysed using the NimbleScan software package in order to identify significant Ac-Histone binding sites.

Establishing miRNA responses to cellular exposures (using Exiqon's MiRCury LNA array, containing 540 sequences representing 454 known miRNAs) may identify a novel set of biomarkers at the level of regulation of gene expression of toxicologically relevant genes. An

interesting possibility is that miRNAs regulate the levels of expression of genes involved in drug metabolism and response to xenobiotics. On the other hand, both drug and xenobiotics exposure might alter the repertoire of miRNA expression. Moreover, miRNA expression is itself regulated by epigenetic factors such as DNA methylation and chromatin structure and thus could mediate the impact of epigenetic reprogramming in response to environmental exposure on a panel of other genes. Since miRNAs also act by changing chromatin structure, they could be considered as a component of chromatin modification and DNA methylation machineries.

Proteomics: The proposed proteomic approach focuses on early/immediate biological responses such as e.g. phosphorylation and oxidation of proteins, which are not detectable by transcriptomics technologies. The proteomics task has the potential to identify the very initial molecular events following exposure to toxic model substances (repeated doses, dose dependent) and provide kinetic details of affected pathways. Besides the 2D-PAGE approach, we will apply quantitative SRM/MRM strategies to provide unbiased information about phosphorylation cascades. With these data, it will be possible to obtain information on the activated pathways and, in combination with quantitative technologies, also the degree of activation (normalization to protein amount). The identification of related kinase/phospatase pathways will provide novel biomarker content for high throughput methods. In a further step, the first verification of these biomarkers by independent methods (antibodies) will provide the basis for larger scale cross-validation and subsequent application of statistical methods.

Metabonomics: NMR spectroscopy and mass spectrometry (MS) are highly fit-for-purpose as metabolic profiling technologies: both can be used such that they are largely untargeted in the molecular structures that will be detected; both have technical extensions that give further detailed structural information, e.g. multidimensional NMR or MS/MS. However they differ in several key respects: MS is several orders of magnitude more sensitive than NMR; NMR spectroscopy is more analytically reproducible and robust across laboratories. Hence, in this project we will utilise a combined NMR/MS strategy to maximise coverage of the metabolome and to provide the greatest range of structural information.

This project will extend our current knowledge in that it will explore for the first time, in a systematic way, the relationship between the metabolome of human *in vitro* cell systems and exposure to chemicals that cause repeated dose organ toxicity. While large-scale (>30 000 samples) industry-sponsored consortia (led by partner “Imperial College of Science, Technology and Medicine”) have shown that *in vivo* toxicity databases can be established for robust prediction of acute organ toxicity from metabonomic data (*Keun et al., 2004; Ebbels et al., 2007*), the translation to *in vitro* systems of such an approach is largely unknown,

especially in the context of predicting repeated dose toxicities. **DETECTIVE** is particularly timely since metabonomic protocols for *in vitro* carcinogenicity testing have been developed as part of other consortia (Carcinogenomics; *Ellis et al., 2010*) and are ready to be optimised for other cell systems. The consistency of a metabolite structure across cell types and species offers clear advantages to the translation of analytical protocols between models.

Sub-project 4: Integration of biomarkers

The general objective of sub-project 4 is to evaluate the significance of putative *in vitro* biomarkers for heart, liver and kidney toxicity by integration of data obtained from all partners involved in this project. Specific objectives are:

- To provide statistical support with experimental design within the consortium.
- To establish procedures for data collection, data storage and data quality management.
- To perform statistical analysis of raw data and selected biomarkers.
- To analyse interrelationships between *in vivo* data, functional readouts and "-omics" readouts.
- To critically evaluate the significance and potential of the functional readouts as novel *in vitro* biomarkers in the evaluation of repeated dose toxicity.
- To critically evaluate the significance and potential of the "-omics" readouts as novel *in vitro* biomarkers in the evaluation of repeated dose toxicity.
- To integrate functional readouts and "-omics" readouts into a set of *in vitro* biomarkers that are highly predictive for *in vivo* repeated dose liver, cardiac and renal toxicity.

The scientific strategy that will be followed to achieve the objectives of this sub-project relies on bioinformatics and statistical analysis of candidate biomarkers as well as on the stabilisation, verification and selection of final biomarkers.

Bioinformatic and statistical analysis of candidate biomarkers: A first task includes the generation of an electronic data base for raw data, which will be done in close collaboration with ToxBank. Extensions for storage of results of statistical analyses will be implemented. Together with the partners from data-generating work packages in the subprojects 2 and 3, endpoints will be defined and relevant covariates will be identified. Based on such considerations, a database will be set up and interfaces for raw data collection will

be developed. Prior to raw data generation, the partners will be given statistical support with experimental design (e.g. choice of concentrations in dose-response experiments, number of experimental repeats). Entering raw data into the database will be supervised and interfaces to the database for convenient data access will be developed. Simultaneously, a quality check of the submitted experimental data will be performed. Together with the partners in the sub-projects 2 and 3, data will be examined for errors and quality problems (e.g. experimental artefacts in microarrays, high experimental variability, outliers etc.). A subsequent set of tasks focuses on the preliminary selection of appropriate biomarkers. Thus, assay data will be statistically processed in such a way that they can be applied for later biomarker characterisation. Based on this information, lists of putative biomarkers for given types of toxicity will be prepared. These will be submitted to the corresponding work packages for experimental verification and final selection. A last task involves the investigation of the interrelationships between established biomarkers and functional readouts. Well-defined genomic pathways (taken from sub-project 3) will hence be connected to functional data on heart, liver toxicity and kidney, as supplied by sub-project 2. If information on clinical signs is available, the relationship between such signs and confirmed biomarkers will be examined (see also the following section).

Verification, stabilisation and selection of final biomarkers: Prior to the final selection of functional and "-omics" readouts as *in vitro* biomarkers for repeated dose toxicity in liver, heart and kidney, a number of selection criteria will be defined, such as the sensitivity of the assay, specificity, predictive power, and relevance. The acceptance and use of biomarkers for regulatory purposes requires indeed a set of quality evaluations to determine the scientific validity of the proposed biomarkers. Information on the predictivity of the biomarker has to be assessed, as well as the appropriate methodologies to do so. In this context, the sensitivity of any given method is imperative. Standardised protocols and standardised reference substances (in collaboration with ToxBank) will be identified for selected technologies, allowing an effective quality assessment of biomarker methodologies.

Next, all relevant *in vivo* data available for the reference compounds, as selected by ToxBank, will be gathered and compared with the *in vitro* datasets produced under **DETECTIVE** in order to determine the most relevant *in vitro* genes and/or functional readouts that are highly predictive for *in vivo* repeated dose liver, kidney or heart toxicity. All **DETECTIVE** partners, as well as experts from ToxBank, will be actively involved in this critical decision-making procedure. Measurement of the final and most relevant biomarkers will be described in an SOP. In addition, as a means of scientific and statistical verification, the biomarker measurements will be independently done in a blinded fashion in another laboratory according to the established SOP procedures. This independent laboratory will be selected by the consortium during the early phase of **DETECTIVE**.

4.4.5 Innovation

DETECTIVE will have the following innovative characteristics and lead to progress on several key issues:

➠ The project focuses on one single key issue of repeated dose *in vitro* toxicity testing - establishment of predictive, sensitive and specific biomarkers - and will thus be able to concentrate efforts to address this challenge in a systematic and integrative approach, applying a broad panel of functional and "-omics" technologies.

➠ The **DETECTIVE** partners can build on substantial previous experience in the field of alternative testing, and in particular on use of "-omics" technologies to identify toxicological signatures. As from project start, **DETECTIVE** will thus benefit from the access to know-how and databases generated in previous or ongoing projects, e.g. ESNATS or CarcinoGenomics.

➠ 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. Partner UKK (Universität Köln, Universitätsklinikum) has successfully generated iPS cell-derived cardiomyocytes which will be 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.

➠ Transcriptomics has successfully been applied to *in vitro* models of human cells for the purpose of predicting toxicity, e.g. with respect to genotoxicity/ carcinogenicity, organ toxicity and endocrine disruption, in general being able to predict toxicity with an accuracy of above 85% (*Balls and Fentem, 1999*). For innovative biomarker development, **DETECTIVE** will now integrate multiple data streams derived from transcriptomics, miRNA analysis, epigenetics, proteomics and metabonomics 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). For developing novel intermediate biomarkers in combination with *in vitro* endpoints for repeated dose toxicity, such integrations are specific research goals.

➠ Completely new insights in long-term toxic effects on cells are in particular expected from new readouts, such as epigenetics and miRNA.

➠ 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 and will provide, for the first time, the fully automated long-

term effect assessment of cosmetic ingredient application.

➠ The combined approach and thorough integrative data analysis in **DETECTIVE** will enable better understanding and identification of mechanisms of toxicity. Analysis of toxicogenomic data will allow the deciphering of signal transduction mechanisms mediating toxicity to the cells, and their use as sensitive toxicity markers. Also, a verification step of toxicological signatures demonstrating critical levels of dosing that can lead to functional cellular failures, will, for the first time, be systematically be addressed in **DETECTIVE**.

➠ New technologies such as shRNA can support the identification of a key biomarker by knocking down the genes of interest.

➠ 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.

➠ For the verification of the identified biomarkers, **DETECTIVE** will also apply an evidence-based approach: a self-critical reassessment of current practices and evaluation of the thus generated information, to make better use of resources and to increase the quality of results, facilitating their interpretation and flexibly accommodating current and future mechanistic understanding (*Hoffmann and Hartung, 2006*).

➠ In line with this approach, **DETECTIVE** will also evaluate and compare the robustness and predictivity of the used measurement readouts, and thereby not only provide biomarkers of high predictive value, but also be able to provide guidance on how to combine the readouts in order to obtain the highest possible predictivity.

➠ In addition to the evaluation of identified biomarkers, the project will also assess the efficiency of the biomarker identification process. **DETECTIVE** will assess the reliability of the screening pipeline as well as the relevance of identified biomarkers. A well characterised and reliable screening strategy will also contribute to the identification of biomarkers that are species specific. Such concepts will be an added value for the identification of novel human biomarkers that cannot be compared to animal data (lack of golden standard).

➠ The principles of such a strategy for biomarker qualification are applicable to a wide range of other highly relevant target cells responding to chronic exposure of chemicals and that have limited repair and renewal capacity (e.g. neurons).

Overall, **DETECTIVE** will lead to a major breakthrough in the field of *in vitro* toxicity testing, moving toxicology beyond descriptive science and towards mechanism-based prediction.

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

Mark Cronin

4.5.1 Introduction

There is a desire to obtain information regarding the safety of a cosmetic ingredient directly from chemical structure. The vision is a suite of computational techniques that allow the user to input a chemical structure into an appropriate suite of software to obtain relevant information. The purpose of seeking computational alternatives for what is, effectively, a series of highly complex biological effects is two-fold:

- To supply information rapidly when no other source(s) of data are available. This may be to assess the risk of an existing ingredient or determine the relative safety of a new chemical.
- To support further non-animal testing e.g. ranging finding or prioritisation as part of an integrated testing strategy. In the same manner, computational techniques may be applied for the development of adverse outcome pathways (AOPs), i.e. the definition of molecular initiating events and their applicability domains at the start of the pathway.

These computational approaches are expected to be inexpensive and rapidly applicable. By their nature they do not rely on animal testing. To be acceptable, it is anticipated that models should be easily comprehensible and scientifically justifiable, rather than pure statistical models or the “black box” approach.

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 address the requirements of 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 use of *in vitro* data is limited at this time due the problem of extrapolation and relevance. While it is unreasonable to expect a single computational approach to predict the complex series of biological effects underlying repeated dose toxicity, the integrated use of multiple models is expected to provide an alternative assessment strategy.

Expecting a single computational approach to predict such a complex series of toxicity endpoints 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 hypothesis of the **COSMOS** project is to develop synergistic workflows for the prediction of repeated dose toxicity to humans being together the following:

- Relevant and openly available databases of toxicological information suitable to support TTC development, grouping for read-across (as well as (Q)SAR) and PBPK modelling.
- Exemplification of the prediction of exposure concentration and its association with hazard.
- Creation of easy-to-use and transparent tools to lead the user through these individual models.

4.5.2 Objectives

The focus of the **COSMOS** project is to develop an integrated suite of open source and open access computational models to assist in the prediction of human repeated dose toxicity for cosmetics. This suite of models will form a flexible and transparent tool within an integrated workflow. The *in silico* workflows will allow for the prediction of repeated dose toxicity to human through the integration of models based on threshold of toxicological concern, innovative chemistry and physiologically based pharmacokinetics. The workflows 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 are to:

- Collate and curate new sources of toxicological data and information from regulatory submissions and the literature
- Create an inventory of known cosmetic ingredients and populate it with chemical structures

➠ Establish thresholds of toxicological concern for endpoints relating to human repeated dose toxicity

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

➠ Establish kinetic and PBPK models *in vitro*, *in silico* and other relevant data to predict target organ concentrations and long term toxicity to humans

➠ Integrate open source and open access modelling approaches into adaptable and flexible *in silico* workflows using the KNIME technology

4.5.3 State of the Art

There are currently no reliable or validated computational alternatives to predict repeat dose toxicity. The area is also particularly lacking in terms of assessing the safety or otherwise from prolonged low dose exposure of humans to cosmetics. The current knowledge gaps are illustrated and summarised in *Figure 4.21* and described in more detail below.

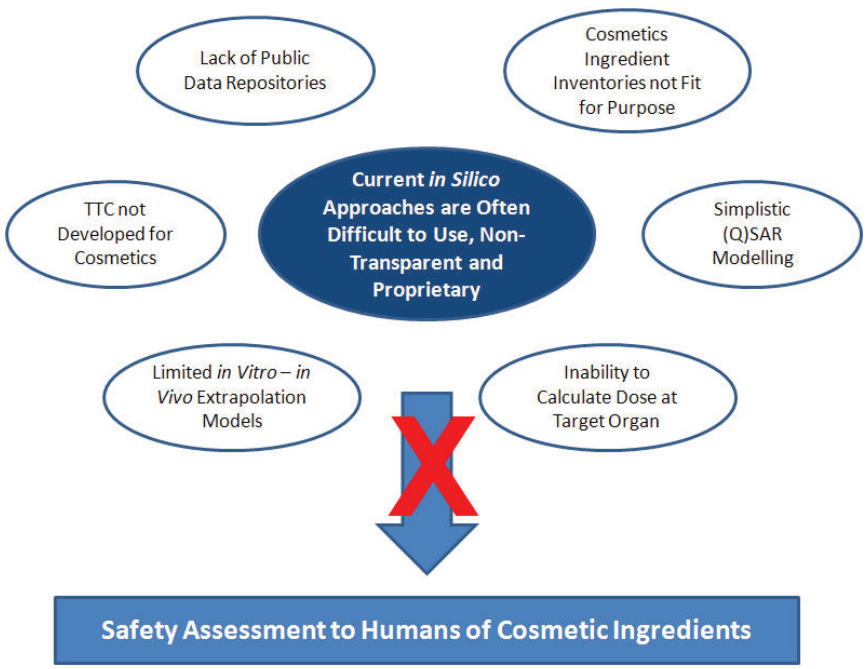

Figure 4.21 *Summary of the knowledge gaps preventing the assessment of the safety of cosmetic ingredients to humans from computational techniques.*

Knowledge Gap 1: Insufficient Toxicological Data and Inadequate Databases

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 are available, for instance RepDose (*Bitsch et al., 2006*) and a database of human Maximum Recommended Therapeutic Dose (MRTD) values for pharmaceuticals (*Matthews et al., 2004*). 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 toxicity endpoints such as repeat dose.

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.

Knowledge Gap 2: Lack of an Inventory for Cosmetics Ingredients Linked to Chemical Structure

The European Commission CosIng database provides a list of over 20,000 cosmetics ingredients with links to SCCS 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 cosmetics 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.

Knowledge Gap 3: Inadequate Thresholds of Toxicological Concern for Exposure of Humans to Cosmetics

The Threshold of Toxicological Concern (TTC) 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 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 (1996)*, who described the development of distribution of NOELs for over 600 chemically diverse substances. These chemicals were sorted in the three Cramer classes (*Cramer, 1978*) and the 5th percentile NOEL was calculated for each. A TTC limit was then established for each Cramer Class by applying a 100-fold uncertainty factor and multiplying by an assumed 60 kg body weight

to put the TTC limit in units of µg/day. The current tiered TTC approach takes this process one step further such that the threshold value can be set for chemicals lacking a full safety profile (*Cheeseman et al., 1999; Kroes et al., 2004*). This tiered approach is presented as a flow diagram that starts by describing groups of chemicals for which TTC should not be used (e.g., non-essential metal or metal-containing compounds, poly-halogenated -dibenzodioxins, -dibenzofurans, or –biphenyls). It then identifies a small group of highly potent carcinogens (which also require a chemical specific risk assessment), and sets a conservative limit of 0.15 µg/d for chemicals with alerts for DNA reactivity that could be associated with genotoxicity/ mutagenicity. For chemicals lacking these alerts and/or having favourable genotoxicity data, the flow diagram moves to higher exposure tiers for non-cancer endpoints (this includes one tier for organophosphates and then the three tiers for the three Cramer classes). The evolution of these approaches has been based for more than two decades mainly on two existing datasets, i.e., the JECFA dataset using no effects level (*Munro, 1996*) and the US FDA dataset with TD50 values selected from the Carcinogenicity Potency Database, which has been expanded to include > 700 chemicals). The approaches have been evaluated to expand to cosmetic ingredients (*Kroes et al., 2007*). Most recently, an attempt has been made to further refine the TTC limit by adding genotoxicity data (i.e., Ames data) for chemicals with genotoxic structural alerts and considering the appropriate duration of exposure (*Felter et al., 2009*). Whilst these are a firm starting point for cosmetics TTC, there is an appreciation that this approach requires updating and development using data for repeat dose exposure to cosmetics.

Knowledge Gap 4: Poorly Developed *In Silico* Models to Predict Repeat Dose Toxicity

In silico methods are computational methods that can be used to predict the physicochemical and biological properties of molecules. They include several approaches, such as read-across, grouping, category formation and (quantitative) structure-activity relationships ((Q) SARs). QSAR estimates have been used routinely for predicting key environmental fate parameters and for ecotoxicological endpoints; increasingly their relevance is being seen for human health endpoints.

There are particular issues with the use of QSAR methods for endpoints such as no observable adverse effect level (NOAEL) or concentrations (NOAEC). QSAR works best for highly defined systems, preferably from a steady-state measurement. It is also true that QSAR should be developed from a mechanism of action viewpoint. Unfortunately, multivariate modelling of NOAEL data does not take account of organ level effects, or the relative mechanism of action. Further, the precise definition of mechanism of action has yet to be made to many organ level effects.

For human health effects, non-testing methods have rarely been used, and where they have

been used, it is generally in the form of grouping rather than QSAR (*Enoch et al., 2009*). Currently the cosmetics industry is working on four priority areas: eye irritation, genotoxicity/ mutagenicity, skin sensitisation and systemic toxicity. *In silico* approaches are used in the cosmetics industry for the prediction of these endpoints; identification of reliable software packages that can be supported over the long-term is now a necessity. At present TOPKAT, a commercial software prediction package capable of predicting chronic oral toxicity (LOAELs following more than 12 months exposure), and Molcode Toolbox are the only commercial tools available. Currently there are no, or only very limited, freely available models for repeat dose toxicity.

Knowledge Gap 5. Absence of Methods to Predict Target Organ Dose

Tools for predicting the target organ concentrations and accumulation of chemicals and their metabolites are currently lacking. In order to extrapolate from *in vitro* to *in vivo* organ level dose, no workflows or integrated systems are currently available. This situation is further 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 that could be 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 physiologically-based pharmaco-kinetic (PBPK) models as well as further development of metabolic simulators.

Knowledge Gap 6. Inadequate Methods to Allow for Extrapolation from *in vitro* Dose to the Concentration *in vivo*

It is apparent that being able to extrapolate from the concentration tested *in vitro* to a likely *in vivo dose* will be crucial to the success implementation of many of the methods being developed in other projects in the **SEURAT-1** Research Initiative. This will be vital to ensure the safety of cosmetic ingredients.

Knowledge Gap 7. No Computational Tools to Allow the User to Link Together all Predictive Technologies in a Transparent Workflow

It is true to say that there are many more users of computational toxicology tools than software and model developers. Therefore the users, who may have a range of expertise from toxicology, risk assessment to chemistry, require user-friendly tools. Another priority is that these tools must be open and transparent, so that they comply with the OECD Principles for

the Validation of (Q)SARs and so that the user can understand how a particular prediction has been made. Current tools have evolved from a predominantly commercial basis (with some notable exceptions such the software from the US EPA and OECD). There is no reason to discourage commercial development of software – it could be extremely damaging to progress and innovation to do so – but the emphasis of the **COSMOS** project is on open access to tools and workflows.

4.5.4 Approach

The general structure of the **COSMOS** project is shown in *Figure 4.22*. The specific approaches are described below.

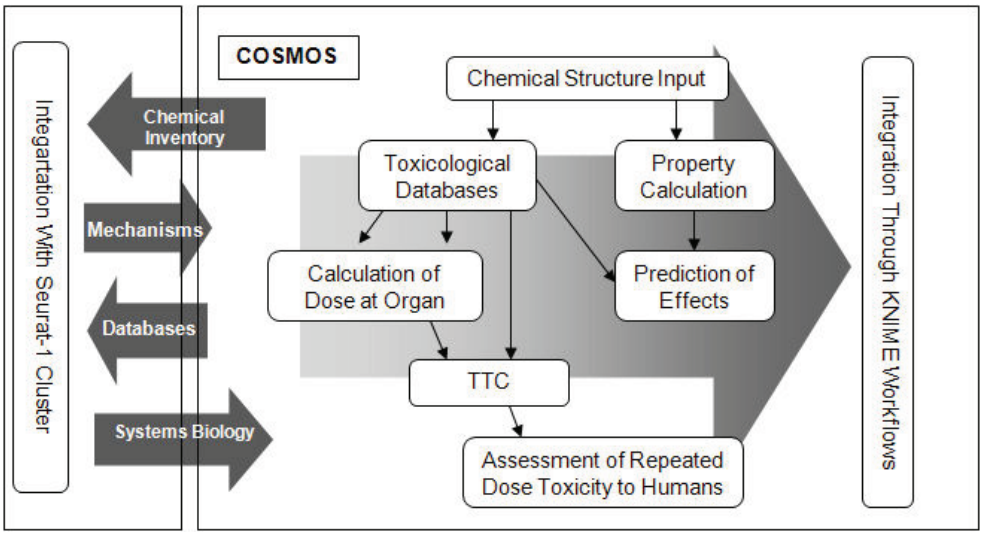

Figure 4.22 *General structure of the COSMOS project including likely areas of interaction with the* ***SEURAT-1*** *Research Initiative.*

Approach to Address Knowledge Gap 1: Insufficient Toxicological Data and Inadequate Databases

The starting point for collecting data has to be to create an suitable database that is adequate, fit for purpose and future-proof. It has to have the ability to capture toxicological data and supporting information and present them in a flexible and searchable format. These data will be captured in logical three-tier architecture (user interface, representation functionalities and database structure and access in addition to Create, Read, Update and Delete capabilities). These will build on existing approaches (e.g. the CERES project at the FDA) and facilite further

data exchange with related or subsequent structures (e.g. ToxBank, OpenTox, KNIME). This will require the development of new data schema and a new User Interface (UI) to accommodate various user levels for accessing data. For this part of the project, additional software tools will be developed, building on those from existing projects (e.g. JRC software development projects, eTox, FDA CERES) and the MOSES molecular editor. Their main specification is that the data repositories will be completely open i.e. will not require other commercial or open source software to be used for search and retrieval. The information within the databases will be curated and its quality assessed according to accepted current criteria. It is important to stress that in the **COSMOS** database will be an open source database tool that will allow full integration with the ToxBank Project.

Approach to Address Knowledge Gap 2: Lack of an Inventory for Cosmetics Ingredients Linked to Chemical Structure

An early goal of the **COSMOS** project will be to create an inventory of chemical structures associated with cosmetics ingredients. Current inventories, such as the EU CosIng inventory, list in excess of 20,000 ingredients. However it is not necessarily comprehensive and, more importantly, is not linked to definitive chemical structures or toxicological information. Therefore, data sources will be searched, which include literature, regulatory agencies, or trade associations, and the content can be either public or proprietary. Sources can be existing off-the-shelf databases or legacy data that require harvesting through data entry activities. Legacy data sources include the US FDA as well as numerous others, such as EU regulatory submissions for cosmetics, OECD / SIDS dossiers, and publically available data sources. US FDA are also able to provide a large list of cosmetics chemicals available through a US voluntary cosmetics registration program (VCRP). In addition, this part of the project will coordinate with the Personal Care Products Council, Colipa, and regulatory agencies to make oral repeated-dose data for cosmetics available for other projects. Also included will be data from existing public databases from US FDA and EPA as well as off-the-shelf databases such as the CPDB (Carcinogenicity Potency Data Base) and Munro datasets used for the derivation of the current TTC approaches.

Approach to Address Knowledge Gap 3: Inadequate Thresholds of Toxicological Concern for Exposure of Humans to Cosmetics

An improved TTC approach will be developed to replace repeated dose toxicity animal testing in human safety assessment. The method development specifically includes the following objectives: 1) to improve and adapt the TTC concept to the human use of cosmetic ingredients; 2) to integrate into the tiered TTC scheme, bioavailability (ADE properties) and human metabolism knowledge; 3) to validate the newly improved TTC approach for cosmetics with test cases, in collaboration with Colipa members and regulatory agencies; 4) to develop

a software application to deliver the new method.

The **COSMOS** TTC dataset will be constructed from the **COSMOS** database. To expand the current tiered TTC approach to cosmetics ingredients to assess human safety, ILSI Europe and US FDA will participate in **COSMOS**. The **COSMOS** TTC dataset will be used to review the appropriateness of relevant data, establish thresholds, and further expand the TTC tiered categories. TTC thresholds will be stratified across the phenotypes and chemotypes such that toxicological concerns can be profiled by the concern factor, defined as exposure divided by threshold. TTC structural categories will be also used for read-across grouping as well as the mode of action classes for QSAR prediction of toxicity. The approach to developing TTC in the **COSMOS** project is summarised in *Figure 4.23*.

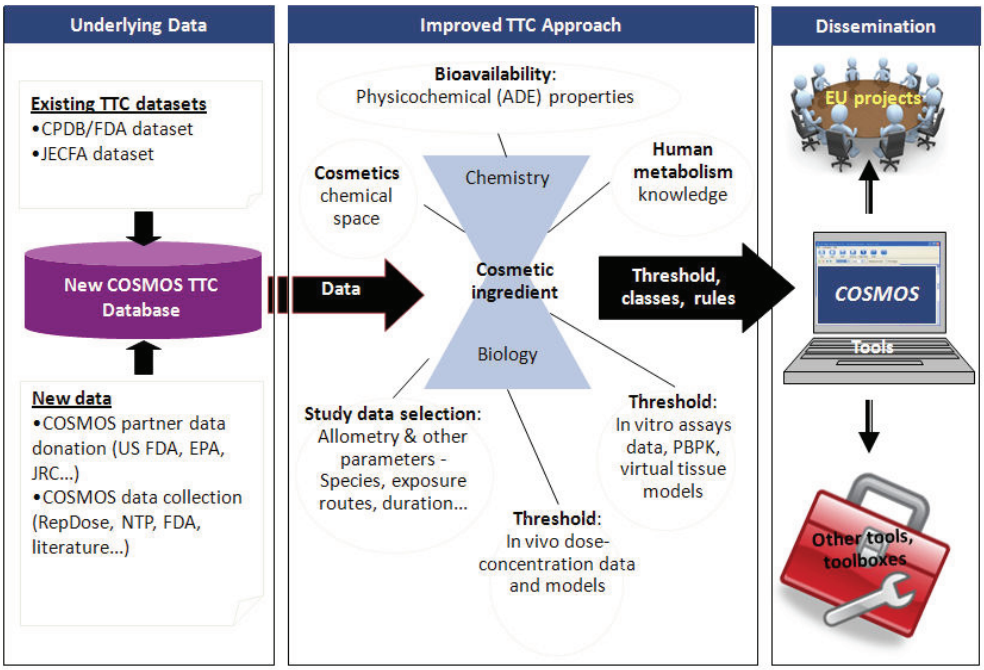

Figure 4.23 *Summary of the development of the TTC approach for cosmetic ingredients as applied in the* ***COSMOS*** *project.*

Approach to Address Knowledge Gap 4: Poorly Developed *In Silico* Models to Predict Repeat Dose Toxicity

There will be a considerable effort to optimise *in silico* methods, such as (Q)SAR, grouping and read-across, for the purpose of long-term toxicity prediction of cosmetic ingredients. This will be performed in conjunction with the characterisation of chemical space of the cosmetic

inventory (see above, Knowledge Gap 2). A dataset containing physicochemical properties, structural information, and *in vivo* data will be available from the **COSMOS** database. This will be used to compare different approaches such as read-across, grouping and QSAR models. Chemicals in the inventory will then be grouped into categories by using freely available software e.g. OECD QSAR Toolbox, Toxtree.

These *in silico* methods will also be employed to refine structural categories such as the Cramer rules as a grouping scheme. Furthermore, the information on the biological profile of the chemicals will be considered in similarity analyses. 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 cosmetic inventory. The search will cover the literature as well as an available database of QSARs models, e.g. the JRC QSAR Model Database, and it will allow the identification of reliable software packages that can be supported over the long-term.

Filling this knowledge gap will also require the **COSMOS** project to embrace new ways of thinking such as the development of Adverse Outcome Pathways (AOPs). An AOP for liver toxicity is illustrated in *Figure 4.24*. The key part for **COSMOS** is the definition of the molecular initiating event and the possibility of using this for chemical grouping and read-across.

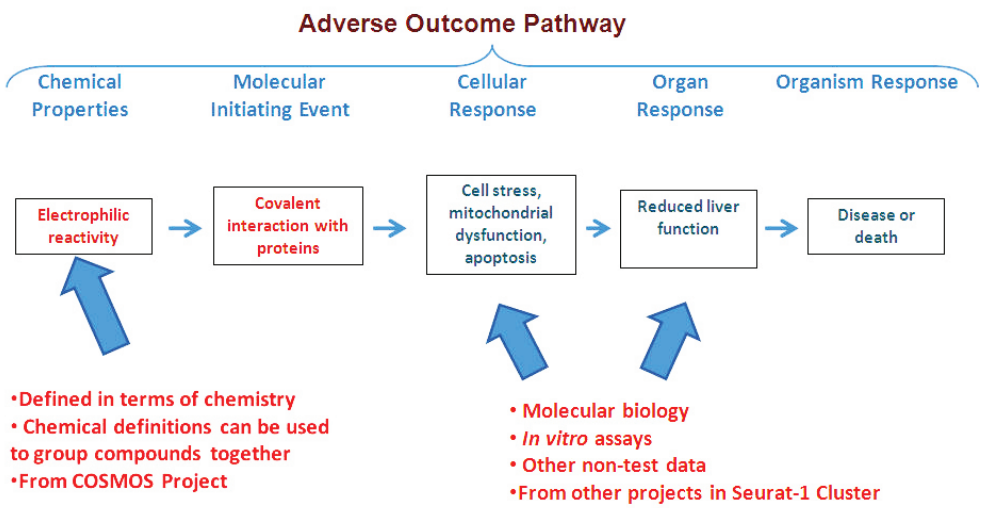

Figure 4.24 *Schematic of a "generic" Adverse Outcome Pathway for liver toxicity.*

Approach to Address Knowledge Gap 5. Absence of Methods to Predict Target Organ Dose

PBPK models will be designed to incorporate information from *in vitro* tests and *in silico* methods developed within the project, in particular relative to metabolism, process-based dynamics models, and *in silico* organs. In particular, PBPK models will integrate the results relative to distribution, cell-lines and quantitative treatment of reactivity and metabolism of chemicals. The software packages isoCYP and MetaboGen developed by Molecular Networks for the prediction of metabolites will be extended to cover also the metabolism of cosmetic ingredients at organ level. PBPK models will also integrate *in silico* spatio-temporal organs. A generic scheme for kinetics modelling is shown in *Figure 4.25*.

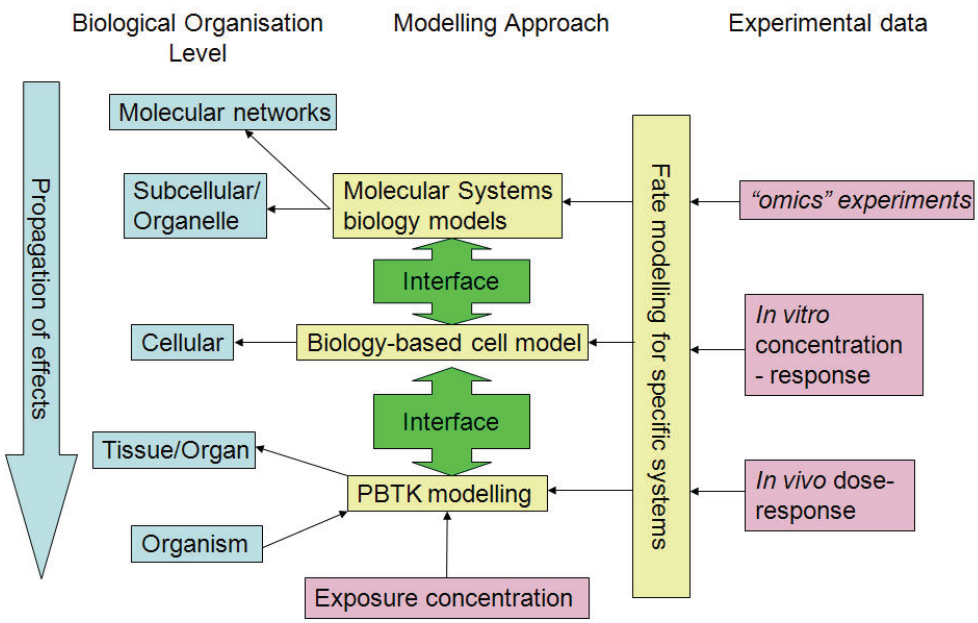

Figure 4.25 *Generic process for kinetics modelling in the COSMOS Project.*

Generic long-time PBPK models (including target organs of interest) will be implemented in cosmetics' risk assessment to humans. A PBPK model based on a detailed compartmentalisation of the body and calibrated with relationships describing the time evolution of physiological and anatomical parameters has already been developed for humans at INERIS (*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 the absorption, distribution, metabolism and excretion 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.

A mechanistic, genome-based 2D *in silico* liver model will be developed and integrated into the PBPK model. The liver model aims at adequately describing the metabolism at a spatial-temporal resolution depending on the substrate-influx provided by the PBPK model. The predicted product-efflux of the *in silico* liver model will be fed back into the PBPK model, thereby enabling genome-based predictions of the long-term effects of potential toxicants. A 2D setup will be applied for constructing the *in silico* liver model: Partial differential equations and ordinary differential equations will be used for simulating the flow of substances and the intracellular network dynamics along the liver, respectively.

PBPK models will also consider existing *in vivo* datasets for selected case studies, and compare the extrapolation from animals to humans with the results used to extrapolate to humans. An assessment of several exposure routes focusing on oral and dermal exposure will be carried out. We will also analyse how *in vitro* and existing *in vivo* data for one or repeated dose exposure could be integrated through the use of models for long-term toxicity prediction of cosmetic ingredients.

Finally, the models will interface with the approaches developed elsewhere in **COSMOS**, through the coupling between TTC and cosmetics ingredients doses at target organs level. Also required will be the new QSAR and chemometric tools developed in **COSMOS** for the estimation of physico-chemical properties, kinetics, metabolism and model parameters – this will be particularly important to address Knowledge Gap 6. These models will produce estimates of the internal concentrations in organs, tissues and cell-lines that will be used to validate the approaches concerning TTC and integrated in a general modelling/flow framework. A general modelling framework architecture compatible with KNIME will be developed.

Approach to Address Knowledge Gap 6. Inadequate Methods to Allow for Extrapolation from *in vitro* Dose to the Concentration *in vivo*

The solution to Knowledge Gap 6 is closely linked to addressing Knowledge Gap 5; whilst they are different problems, there is a great potential for overlap of methods and sharing of resources. *In silico* approaches will be refined to incorporate kinetic and metabolic studies to permit quantitative interpretation of results in terms of consumer risk. In conjunction with metabolic profiles, long-term effects of cosmetics will be quantitatively studied. Metabolic rules will be analysed as part of the molecular descriptors for prediction of chronic toxicity. QSAR models aimed at predicting K_m, V_{max}, partition coefficients or other relevant parameters upon which PBPK modelling is based will be developed based on target organ / *in vitro* effects. These models will be developed for specific groups of chemicals and evaluated for their reliability.

Therefore, existing *in vivo* data as well as *in vitro* data will be used to predict dose in humans following repeated dose exposure. In addition a generic long-time PBPK models (including target organs of interest) for various species and spatio-temporal organs from *in silico* cells will be developed.

The strategy here 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.
- *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 lines
- Physiological based pharmacokinetic (PBPK) models.

Approach to Address Knowledge Gap 7. No Computational Tools to Allow the User to Link Together all Predictive Technologies in a Transparent Workflow

COSMOS will use and enhance KNIME, a modular integration platform to allow the reuse, deployment and archive of data processing, analysis, and prediction protocols as KNIME workflows. KNIME was chosen because its open source nature will allow the dissemination of the project results without charging others. It is already heavily used within many pharmaceutical and biotech companies and KNIME.com already has extensive experience with the integration of other tools and data sources whilst ensuring full security. An illustration of a KNIME workflow to predict the effects of chemicals is given in *Figure 4.26*.

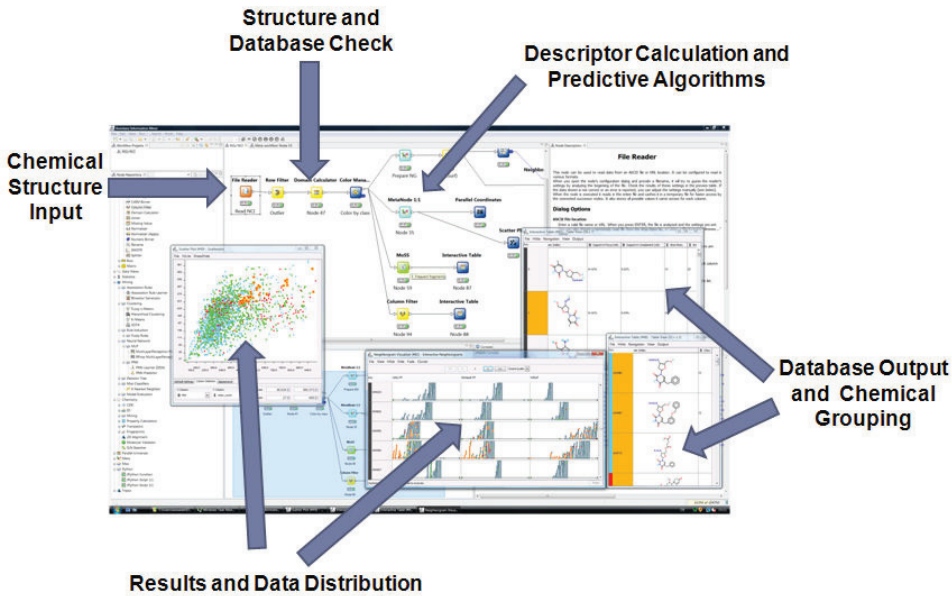

Figure 4.26 *A sample KNIME workflow for predicting effects of chemicals.*

The work in **COSMOS** will require an enhancement of the KNIME modular integration platform. The aim here is to result in an open access and transparent tool for integrating the data with predictive models such that the user will be able to transparently predict repeated dose toxicity from the outputs of the **COSMOS** project. The **COSMOS** project will require the necessary extensions to KNIME allowing additional data sources and tools to be integrated; assist partners in modelling their protocols as KNIME workflows; setup an archival framework allowing reproducible execution of workflows, and setup an infrastructure for remote access to the stored workflows.

Challenges and Risks in the Approaches to Address the Knowledge Gaps

In an ambitious project such as **COSMOS**, there inevitably are numerous challenges to take account of. The greatest challenge will be to engender a change in the way of thinking of classic toxicology to accept, where appropriate, the modelling attempted in **COSMOS**. This acceptance must include an appreciation of the limitations of all models. To overcome this challenge the **COSMOS** project will focus on achievable and high quality science, with demonstrable benefits throughout the lifetime of the project. Appropriate dissemination and training is key in this regard.

There are clear and identifiable risks associated with the **COSMOS** project. These include the following:

➠ Risk: There is currently a lack of an open access database for storing chronic toxicity data; lack of such a database could make collation of data meaningless.

Solution: A new database structure will be developed in **COSMOS** based on a currently accepted model. Issues regarding ontology will be addressed with regard to on-going activities within the **SEURAT-1** Research Initiative as well as outside of it.

➠ Risk: There are few recognised sources of repeat dose toxicity data publicly available.

Solution: New sources of toxicity data are being identified, such as through the US FDA and Colipa members.

➠ Risk: The current TTC paradigm is inappropriate for use for cosmetics.

Solution: The TTC approaches will be updated with the particular input of an ILSI-EU Expert Group.

➠ Risk: Traditional QSAR approaches have been relatively unsuccessful in modelling repeat dose toxicity.

Solution: The emphasis of modelling will be on a mechanistic basis, taking particular account of the adverse outcome pathway (AOP) approach (e.g. from OECD) to guide the grouping of chemicals.

➠ Risk: Extrapolation from *in vitro* to human effects may not be possible.

Solution: Human metabolic cell lines will be utilised for modelling purposes.

4.5.5 Innovation

COSMOS is an innovative project aiming to address the needs of the cosmetics industry through the development of novel solutions. Innovations in the **COSMOS** project come from the following:

➠ A desire to create open source and open access solutions to create computational tools. This will ensure the resources created are available for all to use and apply.

➠ Collation of toxicological data to support the development of TTC and grouping approaches.

➠ Underpinning of the mechanistic basis of toxicity prediction through reference to Adverse Outcome Pathways.

➠ Applying new solutions to predict relevant kinetics parameters to estimate the dose at target organ and allowing for extrapolation from *in vitro* dose to *in vivo* exposure.

➠ Use of novel and innovative software solutions from databasing and data governance, to the assessment of the chemical space of cosmetics ingredients. Combining all models in KNIME computational workflows to ensure easy application and uptake of the models.

➠ Ensuring project integrates with progress with ToxCast and Tox21 by active involvement of appropriate agencies.

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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

The forthcoming complete ban on animal testing in Europe for cosmetic products and the lack of validated alternative assessment methods for long-term systemic toxicity testing have emphasised an urgent need to develop better prediction models in this area. We propose an integrated multifaceted experimental and computational platform using a systems biology approach for long-term toxicity prediction on the molecular, cellular and tissue levels. The experimental work is focused on the application of cellular systems that come closest to human *in vivo* situation, while at the same time allowing their transfer into applicable and easy to handle test systems. In these test systems viability and physiological toxicity response parameters ('-omics') will be monitored together with genetic, epigenetic and structural characteristics in parallel. Large-scale network models of regulatory and metabolic pathways and cellular systems, together with bioinformatics integration of human and across species literature data will lead to reliable toxicity prediction. In the **NOTOX** initiative, we have assembled experts for *in vitro* test systems together with the 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 ^{13}C 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. Various hepatic cell lines will be used in long-term membrane, spheroid and sandwich cultures. As

available, human target cells and organ simulating devices from other projects (see previous project descriptions of *SCR&Tox* and *HeMiBio*) of the **SEURAT-1** Research Initiative will be implemented.

The organotypic model systems will be exposed to repeated low dosages, in a long-term setup, of selected test compounds with known toxicity and future industrial relevance. These compounds will be selected in close cooperation with the integrated data analysis and servicing project of **SEURAT-1** (ToxBank, see following chapter). 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.

Development of 3D tissue structures, cell-cell contacts and intracellular structural features will be characterised by light and confocal microscopy and 3D cryo-electron tomography. 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 above-mentioned technologies, will be analysed by bioinformatic methods. Data from databases, literature, experiments and simulation will be integrated through bioinformatic 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 basis 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 grid computing for analysis and exploration of these models.

4.6.2 Objectives

Major objectives of **NOTOX** are:

- Supplying a versatile methodology for systems based analysis and prediction of long-term toxicity of test compounds on organotypic 3D cultures.
- Development and application of experimental and computational methods for continuous, non-invasive and comprehensive physiological monitoring (respiration, metabolomics, fluxomics, proteomics and peptidomics, epigenomics,

transcriptomics, viability and toxicity reporters, cellular toxicity models) of organotypic test systems upon exposure to selected test compounds.

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

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

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

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

4.6.3 State of the Art

The European legislation requires proof of safety of consumer products on the European markets. A major group of consumer products is cosmetics, which is regulated by the European cosmetic legislation that will impose a complete ban on animal testing in Europe for cosmetic products and individual cosmetic ingredients after March 11, 2013. In addition, the European commission has been encouraging alternative methods for safety assessment of pharmaceuticals, chemicals and other non-food products. For the safety and risk assessment, alternative methods to animal testing based on the principles of reduce, refine and replace animal tests are highly emphasised as has been reviewed by *Pauwels and Rogiers (2009)*. Various initiatives supported by ECVAM have now provided a range of validated alternative methods (*Hartung et al., 2003*). However, the replacement of *in vivo* testing for systemic repeated dose and long-term toxicity in humans still represents a major challenge. 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. This is a necessary prerequisite for such type of testing. On the other hand, a whole toolbox of highly developed analytical techniques, often called '-omics', is now available. This allows a comprehensive characterization of many aspects of cellular behaviour related to toxicity. However, the overwhelming amount of generated data will eventually be effectively used only if data generation and data evaluation are guided by suitable mathematical modelling

activities. Mathematical modelling of cellular and organoid type systems should comprise a detailed physiological description (gene expression, signalling, regulation and transport) and a description of the development of tissue or organotypic structures as a result of exposure to test compounds. Moreover, mathematical models should be used to integrate information obtained from iterative cycles of model predictions and experimental validations in *in vitro* experiments to eventually predict possible toxicity of test compounds *in vivo*. For this purpose, histological human data can be used to provide *in vivo* tissue architecture information and thereby complement the information obtained from the *in vitro* settings. Experimental data, modelling results, as well as databank and literature data, will eventually be combined in large-scale bioinformatic systems extracting knowledge concerning long-term toxicity and its prediction.

Cellular systems

Targeted systemic toxicities include organs such as liver, kidney and brain (*Hartung et al., 2003*). 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 huge and diverse system of enzymes. Of the many human metabolising enzymes, the cytochromes P450 (CYPs) are responsible for the metabolic conversion of most xenobiotics and other exogenous compounds whose elimination is facilitated by metabolism. During this conversion, toxic intermediate products can appear. Other important metabolising enzymes are the conjugating ones, such as the UDP glucuronosyltransferases, other Phase II metabolising enzymes and flavin monooxygenases (FMOs) (*Rendic and Di Carlo, 1997*). In addition to metabolic issues, the role of transporters in the hepato-biliary disposition has been recognised (*Chandra and Brouwer, 2004*). The transporter proteins may be important for the clearance and elimination of the compound when it passes the liver. Numerous transporters are available on the sinusoidal (blood) side of the hepatocyte to mediate uptake of compounds from the blood as well as their excretion back into the blood stream. Hepatic transporters may also play an important role in the excretion of compounds and their metabolites from the hepatocyte into the bile. Compound metabolism is linked to the carbon and energy metabolism of the cells. Toxic effects and changes in compound metabolism are related to these mechanisms.

In addition to human liver cells, mouse organotypic 3D cultures look very promising. In collaboration with the laboratory of Hans Clevers (Hubrecht Institute, Utrecht), **NOTOX** partner “Stichting Het Nederlands Kanker Instituut” (NKI) has demonstrated the presence of approximately six cycling Lgr5+ve stem cells at the bottom of a small intestinal crypt (*Barker et al., 2007*). They have established long-term culture conditions, under which single crypts undergo multiple crypt fission events, whilst simultaneously generating villus-like epithelial domains, in which all differentiated cell types are present. Single sorted Lgr5+ve stem cells

can also initiate these crypt-villus organoids (*Sato et al., 2009*) as depicted in *Figure 4.27*. NKI have obtained the same organoids for stomach stem cells (*Barker et al., 2010*) and also achieved this more recently with liver stem cells.

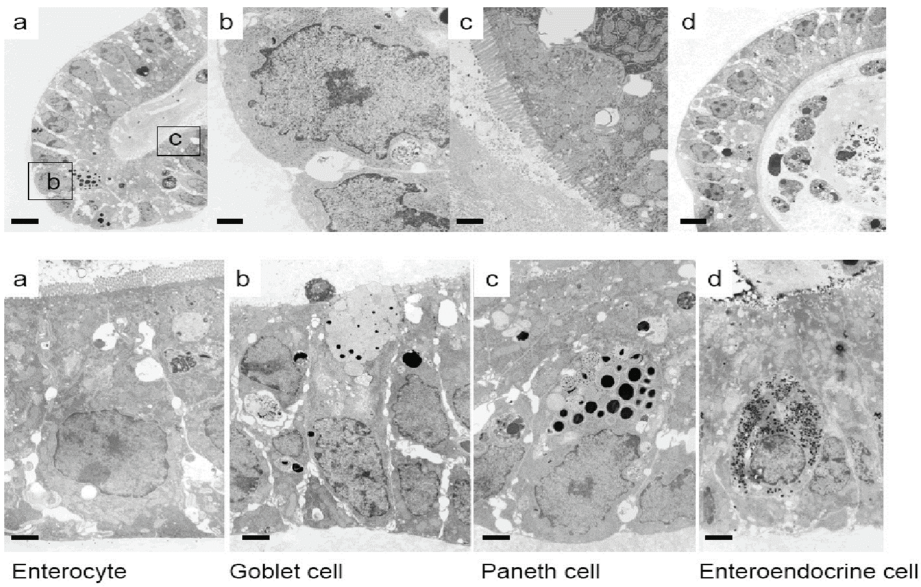

Figure 4.27 *Composition of single stem cell-derived organoids. Electron microscopy demonstrates enterocytes (a), goblet cells (b), Paneth cells (c) and enteroendocrine cells (d) (Sato et al., 2009).*

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 variation. Cell lines can be used with evident advantages of their availability and stable phenotype, however, the majority of the hepatoma cell lines, most commonly used today, contain only low levels of enzymes and transporters necessary for the heptatotoxic drug actions (*Donato et al., 2008*). Therefore, there is also 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*). An *in vitro* hepatotoxicity screening model, therefore, requires cultures containing different cell types of the liver to reliably mimic drug-mediated hepatotoxicity *in vivo*.

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*).

3D membrane bioreactors

A novel and 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 is possible to study metabolism and toxicity because the cells maintain their phenotype for very long times. Intact liver structures including bile ductile-like structures are formed during cultivation. The bioreactor cultures display many of the characteristics essential for studying both metabolism and toxicity in the liver, including long-term maintenance and inducibility of drug metabolizing systems and extended maintenance of other liver specific functions, such as protein and amino acid turnover (*Guthke et al., 2006, Mueller et al., 2011*).

'-omics' technologies (Toxicoepigenomics)

Toxicogenomics: A first characterization of toxic effects may include analysis on the level of transcription (gene expression). However, the scientific community has been sceptical concerning the quality and relevance of transcriptome studies in *in vitro* systems. An example are hepatocyte *in vitro* systems, where culture conditions have been reported to cause massive alterations in gene expression patterns compared to the *in vivo* situation (*Hewitt et al., 2007*). However, recently critical improvements in culture conditions have been achieved, that allow the generation of gene expression data in hepatocyte *in vitro* systems that show a high degree of similarity to liver *in vivo* (*Schug et al., 2008; Godoy et al., 2009*).

Toxicoepigenomics: Epigenomics is rapidly developing as a key discipline in functional genomics. Epigenetic signatures such as histone modifications and DNA methylation are major indicators of functional (physiological and gene regulatory) changes of genomes during development, differentiation and also complex diseases, most likely caused by environmental cues. Epigenomic research will significantly contribute to i) the general understanding of biological systems and ii) the development of new diagnostic tools and molecular understanding of human diseases. The last years have seen an extremely rapid development of comprehensive epigenomic mapping technologies and computational epigenomics approaches. These developments provide an opportunity to use epigenomics data as part of an integrated systems biology approach as outlined in **NOTOX**.

The systemic epigenetic response of organisms and cells to toxic environmental substances such as metals (cadmium, arsenic, nickel, chromium, and methyl mercury), peroxisome modifiers (trichloroethylene, dichloroacetic acid and TCA), air pollutants (black carbon and benzene), and endocrine-disrupting/reproductive toxicants (diethylstilbestrol, bisphenol A and dioxin) have been reviewed by *Baccarelli and Bollati (2009)*. The profiling of epigenomic changes represents an opportunity to model and predict toxicity for several reasons. First, epigenetic marks are highly correlated with all levels of gene regulation, and are therefore expected to be highly informative for the characterization of the cell's response to specific low dose toxicological stresses. Second, epigenetic markers and DNA methylation in particular, are very stable, and provide excellent potential markers for practical purposes. Third, DNA-methylation allows determining epigenetic responses at the single cell level. Fourth, novel sequencing based technologies for high throughput and low volume epigenomic profiling are promising to dramatically increase the practicality and statistical power of epigenetic profiles. Fifth, and perhaps most importantly, epigenetics provide the cell with means for creating a "cellular memory", which may express the long-term effects of low dose treatment in an effective way.

Toxicoproteomics: Among other effects, a toxic molecule will affect the protein expression pattern. Transcriptomics will certainly bring information, but proteomic analysis is an ideal complement, since many differences in the processing are not given by RNA level studies. Proteomics is widely used today in pharmaceutical industries to predict possible toxicities. The well-known case of the cyclosporine A toxicity, predicted for a kidney by proteomic analysis (*Aicher et al., 1998*), is the first of a long series of examples. Progress in proteomic analysis during the last decade (new instrumentation and new strategies), now allows determining the "in depth" proteome changes. Not only differences of expression level of proteins are detected, but very minor structural differences in the protein can be detected as well. Changes in the post-translational modification patterns (processing cleavages, phosphorylations, glycosylations etc.) can be detected and quantified. This information can be obtained much faster, making a comparative toxicoproteomic approach possible in a few months for a series of test compounds. Comparing the proteomes of cells grown with and without a toxic compound is made using different well-described stable isotope labelling methods. Relative quantification can also be made using spectral counting methods, where no derivatization is required. In addition to comparative proteomics study, targeted approaches now permit determining modifications of the processing pattern; this is done, for example, by using N-terminal labelling methods (*Gallien et al., 2009*).

The number of proteins identified can be significantly increased, when, during the LC-MS/MS experiments, nanoLC columns with higher peak capacity are hyphenated with a mass spectrometer and analyser with a high resolution MS/MS instrument. Combining several separation methods (sub proteomes, 1D gels, high resolution nanoLC), differential proteins can by identified and validated as biomarker (*Miguet et al., 2009*). In conclusion, provided

proteomic analysis is performed with a wide range of instrumentation and methodology, it is possible to determine differences in the level of expression, the processing and the post-translational modifications of proteins.

Toxicometabolomics / Fluxomics: Toxic effects are usually also reflected in modified cellular metabolic activities, either by decrease or by amplification of pathway activities, or by the activation of alternative pathways. This also has an impact on intracellular metabolite levels, the metabolome. It is reported that even sub-toxic concentrations can cause significant effects in cellular metabolism of HepG2 cells. These changes can be measured by the changes in the metabolite concentrations or, even more clearly, by determining metabolic fluxes (*Niklas et al., 2009*). NMR techniques have been successfully applied to metabolic flux analysis in cancer cells using ^{13}C labelled substrates (*Forbes et al., 2006*) and mass spectrometric techniques are increasingly applied for steady-state flux analysis. Steady-state ^{13}C labelling methods and NMR were applied to measure fluxes in tumour spheroids (*Kim and Forbes, 2007*). Metabolite balancing has been applied for a human cell line to determine dynamics of metabolic fluxes (*Niklas et al., 2011*). Toxic effects of free fatty acids on hepatoma cells were reflected in metabolic flux changes (*Srivastava and Chan, 2008*). Current developments in metabolic flux analysis in eukaryotes are recently reviewed (*Niklas et al., 2010; Niklas and Heinzle, 2011*).

Structural changes

The cell is made up of numerous types of macromolecular complexes or biological nanomachines. These form functional modules that are organised into complex subcellular networks. Information on the ultra-structure of these nanomachines with and without exposure to toxins has mainly been obtained by analysing isolated structures, using imaging techniques such as X-ray crystallography, NMR, or single particle electron microscopy (EM). Yet there is a strong need to image biological complexes in their native state and within a cellular environment, in order to gain a better understanding of the effects of compounds. Emerging methods in EM are now making this goal reachable.

Cryo-electron tomography bypasses the need for conventional fixatives, dehydration and stains, so that a close-to-native environment is retained. As this technique is approaching macromolecular resolution, it is possible to create maps of individual macromolecular complexes. X-ray and NMR data can be ‘docked’ or fitted into the lower resolution density maps to create a macromolecular atlas of the cell under normal and stress (such as exposure to toxicant) conditions. Since organoids are too thick to be imaged using intact state, methods such as ‘high pressure freezing’ with ‘cryo-sectioning of unperturbed vitreous fully hydrated samples’ have been developed for electron tomography by few groups including **NOTOX** partner “Stichting Het Nederlands Kanker Instituut” (“The Netherlands Cancer Institute”;

Pierson et al., 2010). For cryo-electron tomography, 100 images are taken, slowly tilting the specimen through a 140° arc. First, these raw images are combined using tomography techniques resulting in a 3D map of the electron density within the specimen, a 3D view right through the cell. Next, proteins are identified by matching their electron densities to those determined by X-ray crystallography and NMR.

Currently, most common algorithmic techniques for obtaining 3D electron density map from the tilt series are variants of either weighted back-projection (*Gilbert, 1972; Radermacher, 2006*) or algebraic reconstruction techniques (*Herman et al., 1973; Mueller, 1998; Bilbao-Castro et al., 2009*). The former though very fast are sensitive to noise, irregular acquisition geometry, truncated projections, and low-contrast measurements, all of which appear in the cryo-electron tomography settings. Moreover, prior information and acquisition process modelling are hard to include. On the other hand, algebraic techniques though known to produce superior results in terms of quality have extreme computational complexity which limits their use on datasets of practical relevance.

Integrative and predictive computational systems biology in toxicology

Systems biology methodologies for designing novel revolutionary predictive toxicology systems require a multidisciplinary approach and thus a need for very diverse sets of experts (toxicologists, cell biologists, mathematicians, statisticians etc). Various '-omics' based technologies and 3D structure analyses will be integrated in **NOTOX** to generate computational models for repeated dose toxicity assessment. Most systems biology scenarios in toxicology advocate the use of data recently delivered by post-genomic technologies (microarrays, proteomics, etc.). Now more than ever, it is important to capitalise on the vast body of data generated in long-term toxicity testing in animals over the last decades, as well as in molecular biology, biochemistry, pharmaceutical and clinical research.

Modern computational tools that allow systems to be perused 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, proteins in addition to chemicals, permits people in different parts of an organisation to understand things faster, and to speak the same language to some extent. 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, and such models have 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).

Concluding remark

The existing long-term test methods involving animals can only be replaced by incorporating various strategies in an integrated multifaceted platform using a systems biology approach. This will also require *in silico* methods and powerful bioinformatic tools to process huge amounts of data that is usually collected in a typical systems biological setup. This systems approach is highly promising for a more comprehensive understanding and better prediction of repeated dose toxicity of test compounds.

4.6.4 Approach

Using today's state-of-the-art in alternative methods as starting point, the **NOTOX** project will explore and establish beyond the state-of-the-art techniques based on a systems biology approach to establish causal predictive models for long-term toxicity using human relevant test systems.

Cellular systems

In general, it would be most desirable to generate differentiated human cells supply from stem cell lines. Such systems will be developed by *SCR&Tox*. Reliable and homogenous production of differentiated cells, e.g. hepatocytes, cardiomyocytes or neuronal cells from these preferentially embryonic stem cell lines would provide a sound basis for compound testing for human long-term toxicity. Presently the only cell lines available for long-term cultivation are the tumour ones.

In order to obtain an intermediary good *in vitro* system, various different hepatoma cells can be used for toxicity studies. However, most hepatocyte cell lines (mainly originated from tumours) although they have indefinite proliferative capacity, they are considered inappropriate for prediction of hepatotoxicity in preclinical drug development due to the low, if any, levels of major CYP enzymes and several transporters. In general, cell lines like Huh-7 and HepaRG increase their expression of drug metabolizing enzymes when becoming confluent.

A recent study was undertaken within the EU-framework LIINTOP project to compare transcript and activity levels of the major CYPs and their responsiveness to the prototypical inducers, phenobarbital (PB), rifampicin (RIF), and omeprazole (OME), over a 4-week period in differentiated HepaRG cells (*Lambert et al., 2009*). For this purpose, CYP activities were

simultaneously estimated using a cocktail of probe substrates. The obtained results show that basal mRNA and activity levels of major CYPs, expression of several phase II enzymes and transporters, as well as inducibility by RIF, PB, and OME were well-maintained in differentiated HepaRG cell cultures during the 4-week period tested (*Anthérieu et al., 2009*).

The data from this study support the conclusion that HepaRG cells exhibit a drug metabolism capacity, including responsiveness to chemical modulators, and, in addition, offer several unique advantages, including: (i) the data are reproducible during several passages; (ii) the functional activities are well-maintained for several weeks at confluence; (iii) the levels of activities can be modulated by selecting appropriate culture conditions, especially the composition of the culture medium.

Human liver organoids derived from hESC or iPS would be an attractive alternative cellular system for long-term toxicity testing.

Culture techniques

Since the classical 2D-culture techniques are usually not well suited for long-term cultivation, 3D culture techniques have gained a major focus. Organotypic cultures are tissue culture models that mimic *in vivo* tissue architecture through manipulation of different cells of an organ within and on top of an extracellular matrix. A benefit of organotypic cultures is that they incorporate aspects of cell-matrix and cell-cell interactions that cannot be evaluated in monolayer cultures. Organotypic cultures more accurately mimic physiological conditions. Organoids are organ-like bodies 'constructed' in culture from a limited number of multipotent cells and growth factors. Work on organoids has many potential benefits in basic biology and for medicine, and for use in testing compounds, these organoids should be more revealing than single cell types, since they more closely resemble the *in vivo* situation. Various groups use microstructure supported formation of organoid type cultures, e.g. (*Khetani and Bhatia, 2008; Mori et al., 2008; Cho et al., 2010*). Such techniques will certainly be important for future compounds testing.

Sandwich cultures

3D culture of cells can be achieved by sandwich cultures using appropriate extracellular matrix, as depicted in *Figure 4.28* (*Godoy et al., 2009*). Using this 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 (*Ullrich et al., 2007*). Long-term 3D sandwich cultures of hepatocytes will be developed for routine testing of repeated dose toxicity. For this purpose, the initial time periods will be determined after which *in vivo* relevant

concentrations of hepatotoxic compounds cause clearly detectable alterations of the *in vitro* system, using a recently published battery of functional endpoints for *in vitro* hepatotoxicity testing (*Brulport et al., 2007; Hewitt et al., 2007*) and RNA expression profiling.

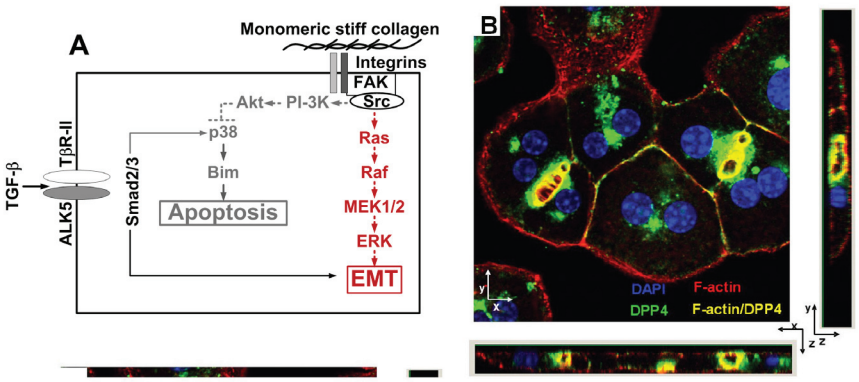

Figure 4.28 *Control of hepatocyte differentiation in vitro by early signalling (from Godoy et al., 2009). A. Using conventional culture conditions Ras/ERK as well as PI-3K signalling are over-activated by constituents of the extracellular matrix, leading to dedifferentiation (EMT: epithelial-to-mesenchymal transition) and apoptosis resistance. B. We have established culture conditions based on optimised extracellular matrix that guarantee in vivo like (low) activities of Ras/ERK as well as PI-3K signalling. Only under these conditions hepatocytes maintain their differentiated functions, such as phase one and two metabolism and a polarised cell state. Fig. B visualises bile canaliculus formation at the hepatocyte interface (yellow).*

Spheroid cultures

Spheroid cultures have been used for a long time, but recently *Brophy et al. (2009)* described 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 cell have been cultured on a gyratory shake 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 (*Figure 4.29*). 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*).

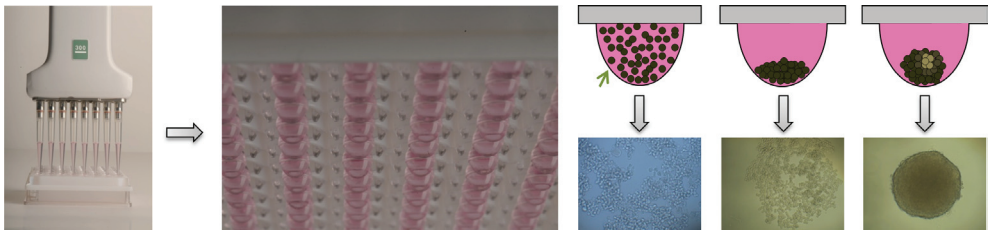

Figure 4.29 *Spheroid formation in microtiter plates using InSphero technique (courtesy of InSphero, Zurich).*

3D membrane bioreactors

The bioreactor model to be used in **NOTOX** allows studying bile drainage, as well as microscopic analysis of particular interest. These multi-compartment bioreactors have three independent fibre capillary membrane systems. The capillary layers are interwoven, which result in a 3D capillary network with repetitive units for cell immobilization. The capillary systems are made of porous membranes that allow medium supply and enables efficient gas exchange (*Figure 4.30*). The bioreactor model shows relevant liver tissue characteristics, and it maintains the appropriate liver functions for long times (*Mueller et al., 2011*). Experiments conducted in the laboratories of **NOTOX** partner "Karolinska Institutet" (KI) reveal that P450 activity remains for up to 2-3 weeks in the best reactors. This bioreactor has been operated for some time already in the labs of KI and the "Saarland University" (USAAR), cultivating HepaRG and primary human hepatic cells, respectively.

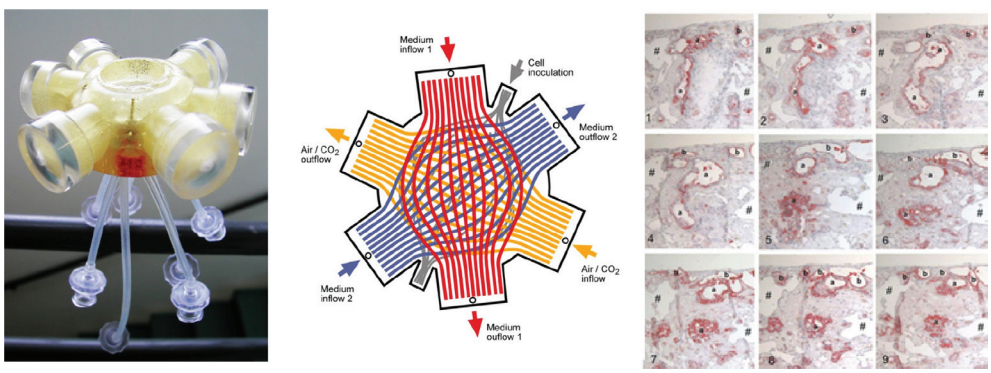

Figure 4.30 *Membrane bioreactor. Left: Miniaturised model of a bioreactor for 1-2 ml of cells. Center: The bioreactor consists of three independent capillary systems, two of them are perfused with medium in counter-current flow and the third serves as oxygen supply. Right: Histological characteristics of the liver like structures obtained in the in vitro bioreactor. Analysis of the 3D-arrangement of bile-duct-like structures in bioreactor cultures. Bile duct-like structures are labelled with anti-CK19 antibod (Schmelzer et al., 2009).*

The cells, such as human liver preparations, can be inoculated and exposed to different test compounds in the absence or presence of inducers and inhibitors. Medium is collected for analysis of test compound metabolites and products. The cell system can be repetitively used after re-equilibration to basal conditions. After culture termination, material can be analysed for gene expression levels at both mRNA and protein levels, as well as for immunohistochemical analysis.

In a different type of membrane reactor, the cells are cultivated inside single hollow fibre membranes. This recently published hollow fibre culturing technique showed high functionality over about 4 weeks (*Mizumoto et al., 2008*). The fibres of 200 µm diameter would be ideally suited for cryo 3D-electron microscope tomography preparations. More recently, the same group described the formation of vascularised liver spheroids using a related technique (*Linke et al., 2007; Inamori et al., 2009*).

Gene expression and toxicity (Toxicogenomics)

A first characterization of cells applied will be on the level of transcription. The comparison of the transcriptome of liver and other cells at different levels of long-term exposure will provide a first indication of toxicity mechanisms. In the bioreactor model, introduce various inhibitors will be introduces. The enzyme inhibitors will conclude the enzyme specificity for metabolic activation of these test compounds. The antioxidants and kinase as well as phosphatase inhibitors will be added to evaluate the participating intracellular signalling cascades involved in the mechanisms for the compound induced hepatotoxicity.

For validation, the effect of selected drugs and chemicals on morphology and the release of soluble toxicological markers will be determined. The bioreactors will be exposed to model hepatotoxins and drugs known to produce hepatocellular, cholestatic or a mixed type of liver toxicity as well as steatosis. The markers that will be studied include alanine transaminase (ALT) 1 and 2, aspartate transaminase (AST), markers of steatosis, cytochrome P450 induction, mitochondrial toxicity, oxygen consumption, as well as albumin and urea production. The dose effect relationships for these drug-induced hepatotoxic effects will be monitored and compared with similar data obtained in single cultures of human hepatocytes, the usual system employed today. Due to the easy drainage of medium from the bioreactors at different time points, we will analyse early biomarkers for drug-induced hepatotoxicity developed at a later stage. Such biomarkers, including cytokeratins, chemokines and various cytokines, have also been identified by **NOTOX** partner “Karolinska Institutet” for alcohol-induced hepatoxicity *in vivo* using microarray analysis (*Butura et al., 2009*).

The global gene expression changes in the bioreactor models at different time points after treatment with the test compound in question will be monitored using Affymetrix 1.1 ST human arrays assessing the expression level of 28,853 genes. Specific changes in the gene

expression pattern will be monitored in response to the various chemicals, while software analysis will provide the information regarding the affected intracellular signal transduction pathways.

Transcriptome analysis relies on intracellular material that is easily obtained in 2D, 3D-spheroid and sandwich cultures. Membrane reactors have to be destroyed to obtain samples. The actual analysis uses well-established methods applying either DNA-chips or real time PCR. The potential drawbacks of this approach are discussed above (see chapter 4.6.3 “State of the art”).

Proteome and toxicity (Toxicoproteomics)

The toxicity will also be monitored using proteomics in the medium, released by the bioreactors. Aliquots of the medium will be subjected to gel filtration or ultrafiltration for removal of albumin and immunoglobulins and other high molecular weight compounds, whereas the lower molecular weight fractions will be subjected to LC/MS/MS. The release of novel proteome based biomarkers for toxicity will be monitored.

The recent development of Multiple Reaction Monitoring (MRM), where only specific transitions are detected, open the door for much faster analysis, once a first proteomic study has detected changes. Up to several hundred peptides can be quantified simultaneously. The MRM methodology now allows high-speed analysis compatible with the identification of toxicity marker molecules.

Epigenome characterisation and toxicity (Toxicoepigenomics)

The **NOTOX** partners “Saarland University” (USAAR) and “Weizmann Institute of Science” (WIS) have a long-standing expertise in epigenomic profiling, particularly the DNA-methylation analysis. USAAR has participated in creating the first high resolution DNA-methylation map of all promoters of a human chromosome by bisulphite mapping (*Zhang et al., 2009*). This partner has established MeDIP profiles of Hep G2 cells along human chromosomes (as a reference map along several human chromosomes and all promoters using array hybridisations (*Figure 4.31* shows a MeDIP analysis image, unpublished). In addition, the group has established a working pipeline to analyse DNA-methylation by bisulphite sequencing on a 454 GS FLEX platform to obtain high resolution profiles at single chromosome resolution of a tissue/cell mixture. WIS is developing algorithms for the analysis of DNA methylation profiles using MeDIP-chip and MeDIP-seq in collaboration with Peter Jones group (*Gal-Yam et al., 2008*). The group has developed new methods for M.SssI based normalization of DNA methylation profiles and had recently derived a detailed sequencing-based genome wide characterization of DNA methylation and Polycomb changes during prolonged *in vitro* transformation.

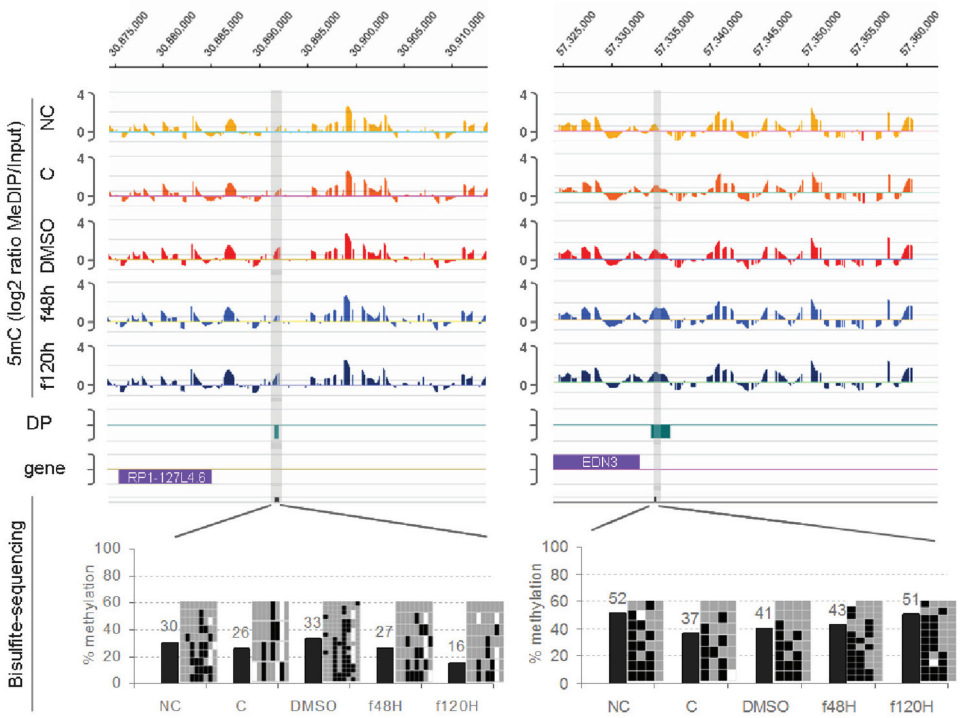

Figure 4.31 *Snapshot of a MeDIP based genome wide methylation analysis of HepG2 cells human chromosomes (Pälmke et al., 2011). The upper panel depicts the identification of areas with differential methylation in cells cultivated following a specific freeze/thaw procedure. The lower panel shows bisulphite validation using bisulphite sequencing (black - methylated CpG-positions, grey - unmethylated CpG-positions).*

USAAR has a long-standing close collaboration with the group of T. Lengauer, MPI Saarbrücken in developing bioinformatics strategies dealing with analysing and predicting DNA-methylation in the genomic context (*Bock et al., 2006; Bock et al., 2007; Bock et al., 2008*). WIS is developing new methodologies for understanding the functional and evolutionary role of mammalian DNA methylation (*Tanay et al., 2007*). In addition, WIS has significant expertise with the development of algorithms for integrating multi-layer epigenomics data (*Jaschek and Tanay, 2009*), and in larger scale integration of heterogeneous system-level datasets and models (*Gat-Viks et al., 2004; Tanay et al., 2004*).

WIS is developing models and algorithms for understanding DNA methylation patterns using theoretical analysis of sequence evolution (*Mendelson-Cohen et al., 2011*) and extensive integration and normalization of epigenetic profiles derived from MedIP-chip/MedIP-seq experiments or deep-bisulfite sequencing (*Gal-Yam et al., 2008*). The group is interested in developing unbiased methods for testing the causal and non-causal emergence of DNA

methylation patterns in tissues and culture models, and had recently derived a detailed sequencing-based genome wide characterization of DNA methylation and Polycomb changes during prolonged *in vitro*. In **NOTOX** both groups will jointly contribute to the epigenomic profiling of cells derived from the organotypic cultures. In brief, DNA and chromatin material will be collected from cells of the organotypic cultures (hepatocytes) at distinct time points following exposure to test compounds. Epigenomic profiling of cells will be performed at various stages on a genome wide scale using MeDIP and ChIP (H3K4 mono and H3K27 tri) combined with sequencing. In order to detect responsive epigenetic changes, the data will be normalised, statistically evaluated, ranked and exchanged between groups for further evaluation. In the first, step DNA-methylation and histone methylation data shall be correlated and compared to reference maps available (e.g. ENCODE data, NIH reference maps, and our own HepG2 map). The data will then be correlated to transcriptome, proteome and metabolome data to identify primary regulatory pathways, affected by epigenetic changes. Several test compounds and reiterated treatments (biological replicas) will have to be tested including positive control substances such as as 5-Aza-cytidine (VIDAZA), 5-aza-2-deoxy-cytidine (DECITABINE) or valproic acid (VPA), known to induce epigenetic effects at low sub-lethal doses.
Upon determination of epigenetic responsive regions to toxic substances, USAAR will perform a deeper analysis of tissue samples by deep bisulphite sequencing, e.g. at CYP450's known to be epigenetically regulated. This will allow a high-resolution determination of epigenetic patterns to (re-) construct the dynamics of epigenetic changes in individual cells of tissues. The resulted dynamics will then be correlated to cellular and 3D changes measured on the same system.

Metabolome/fluxome and toxicity (Toxicometabolomics/fluxomics)

In routine operation, extracellular metabolites, i.e. carbohydrates, amino acids and other metabolites secreted by the cells are measured by HPLC, GC-TOF-MS and LC-MS/MS. Intracellular metabolites will only be measured for special purposes, e.g. dynamic metabolic flux analysis. Dynamic flux analysis by measuring intracellular metabolites and their ^{13}C labelling cannot be done in membrane reactors, since cells are not accessible in these closed systems. However, in spheroid and sandwich cultures, this is generally possible, but methods have to be elaborated. HPLC is usually best for quantification, particularly of amino acids, major media components and secreted organic acids and was used for the classification of drugs according to their mechanisms of action in HL-1 cardiomyocytes (*Strigun et al., 2011*). GC-TOF-MS allows the detection and quick identification of a broad range of compounds with moderate effort, but is limited with respect to quantification and compound spectrum. **NOTOX** partner "Saarland University" applied GC-MS to prioritise host targets for antiviral drug screening (*Schneider et al., 2009*). LC-MS/MS methods provide results complementary to the others mentioned, particularly for compounds that are not easily converted into volatile derivatives.

Metabolic flux analysis can be carried out at different depths. Routine flux analysis can be done on the basis of metabolite balancing (*Niklas et al., 2010*). Steady-state flux analysis can be extended by modified techniques and can show high resolution of several pools of one metabolite in different compartments (*Deshpande et al., 2009; Niklas et al., 2010*). A more detailed resolution of intracellular fluxes requires the application of experimentally and computationally more demanding dynamic labelling experiments, as were recently carried out by members of the **NOTOX** consortium (*Hofmann et al., 2008; Maier et al., 2008*). This method also provides kinetic information, e.g. by applying lin-log kinetics, which will be identified in **NOTOX**. Intracellular metabolic rates will also be measured in organoid systems, based only on the measurement of extracellular metabolite conversions assisted by the application of ^{13}C labelled substrates and later after development of necessary sampling techniques in sandwich and spheroid cultures. Respiration of mammalian cells is indicative of viability and provides information about toxicity effects (*Beckers et al., 2010; Noor et al., 2009; Mandenius et al., 2011; Mueller et al., 2011*).

Structural changes and toxic effects

Cryo-electron tomography for exploring changes in the 3D organization of biological samples (i.e. changes in the cellular ultra-structure) through toxicants will be applied in **NOTOX**. To obtain reliable, high-resolution, and high-contrast 3D maps suitable for resolving the macromolecular structures with desired accuracy and detail, a beyond-state-of-the-art reconstruction pipeline based on parallel Simultaneous Algebraic Reconstruction Technique (SART), is being developed within the **NOTOX** project. The key features are inclusion of prior information about the specimen, hierarchical computation, proper acquisition process modelling including contrast transfer functions and extensive use of many-core computing architectures.

The proposed pipeline decreases the computational time of algebraic reconstruction by an order of magnitude, while also significantly enhancing the quality of the reconstruction, in terms of noise reduction and feature recognition, making the subsequent analysis more feasible. Moreover, due to its robust handling of incomplete projection sets it allows taking smaller amount of measurements, further reducing the radiation damage of the specimen. Once the raw 3D data has been obtained, subsequent analysis is performed to identify the macromolecular and cellular structures including cell-cell contacts, intracellular vesicles or membrane structures.

Commonly, density-based techniques like thresholding with region growing, watershed transform (*Volkmann, 2002*), or level-sets (*Baker et al., 2006*) are used. Increasingly, especially for docking high-resolution X-Ray or NMR data, template-based matching (*Lebbink et al., 2009*) or cross-correlation searches (*Frangakis et al., 2002*) are employed. However, as pointed out by *Sandberg (2007)*, the intensity-only based methods, though fast, are not

robust. The template-based matching, on the other hand, requires comprehensive atlases and is sensitive to bias and target shape changes, especially due to diseases or abnormal deformations.

NOTOX aims at extending these methods with additional information provided by ray casting techniques, geometrical properties, or energy-based segmentation to deliver robust algorithms suited for the low-contrast, high noise setting of cryo-electron tomography. Emphasis will also be put on the use of parallel techniques on many-core architectures and on combination with volumetric visualization and advanced transfer functions to increase both the speed and the informational value of the analysis.

Bioinformatic analysis and Modelling

Computational Epigenomics: The ambitious goal of the computational epigenomics package in **NOTOX** is to create the structures and tools for integrating epigenetic data into metabolomics, toxicology networks and structural data sets and models. This will open a new facet of interpreting cellular responses to toxins. To obtain this goal, the **NOTOX** partners "Weizmann Institute of Science" (WIS) and the "Saarland University" (USAAR) face two major challenges: i) the comprehensive epigenomic data sets have to be analysed at both the primary (normalization, validation) and higher (extracting meaningful features and performing comparative analysis of profiles) levels ii) novel strategies and tools have to be developed to be able to integrate and evaluate epigenomic data in combination with molecular, metabolic and structural parameters.

For primary analysis, USAAR is developing R based statistical tool pipeline for data filtering MeDIP and ChIP data. USAAR also implemented improved algorithms for mapping of bisulphite sequencing data derived from deep sequencing on the basis of BiQ (*Lutsik et al; 2011*). WIS is developing an integrated environment for normalizing and comparing DNA methylation and other epigenomics profiles (*Jaschek and Tanay, 2009; Schuettengruber et al., 2009*). WIS had also assembled a comprehensive collection of existing DNA methylation profiles for reference and is continuously updating it for usage in analysis of new data.

A major challenge in the exploitation of epigenomic markers in **NOTOX** is their integration with data on metabolic, transcriptional, proteomic and structural aspects of the studied assays. WIS will approach these challenges using a novel two-level approach. First, WIS will model epigenomic data and infer a probabilistic model that will describe the epigenomic state using a combination of few model behaviours (e.g., promoter, enhancer, insulator, with or w/o DNA methylation). For this purpose, WIS will integrate **NOTOX** data with reference epigenomic (ENCODE, NIH Roadmap and more). At the second level, WIS will develop a Bayesian network model and other machine learning techniques for studying the interactions between epigenomic variables and other types of variables (metabolic, transcriptional, structural). The

two-level approach will provide novel ways for the integration of data on multiple aspects of biological regulation, and will allow extraction of key epigenomic variables from otherwise almost incomprehensible multi-gigabyte epigenomic profiles. On these integrated analyses, WIS will closely collaborate with partners "Cambridge Cell Networks Ltd." and USAAR to i) determine data formats, ii) build up exchange pipelines iii) ensure data storage and iv) provide exchange of knowledge for data interpretation.

Bioinformatic analysis of '-omics': The integrative analysis of multiple layers of '-omics' information for the derivation of effective toxicity prediction model requires two complementary approaches to be applied simultaneously. First, a comprehensive modelling-based strategy needs to be applied, in order to characterise how different layers of response (metabolic, transcriptional, epigenomics and structural) are correlated. The **NOTOX** partners enjoy a diverse range of expertise to successfully meet this goal, using advanced kinetic modelling techniques, machine learning, sophisticated image analysis and feature extraction, and mad extensive literature mining. Second, the observations on the activity of variables at the different layers of the model, and on the interaction among them, must be transformed into a practical and cost-effective prediction strategy. By greatly expanding the repertoire of potential reporter variables, and by careful analysis of their statistical and mechanistic dependencies, the **NOTOX** approach is ideally poised to identify the most effective predictive measurable, which can then be applied in practice.

Modelling regulatory and metabolic pathways: Quantitative predictions of intracellular and extracellular dose-level dynamics require using mechanistic kinetic network models. Genome-oriented growth/death predictions necessarily rely on dynamic network models capable of simulating the biochemical formation of macromolecules such as (glyco) proteins, polysaccharides, and DNA/RNA, which are the reasons why these network-models are complex by nature. Reconstruction, simulation, and validation of large-scale network models are, therefore, still in their infancy. However, since mammalian metabolic networks operate on a timescale of minutes to hours, predicting long-term repeated dose effects requires accounting for the interaction of metabolic pathways with regulatory networks.

For managing the various layers of complexity, the **NOTOX** partner "Insilico Biotechnology AG" (INSIL) has developed a graphically-oriented setup of metabolic and regulatory/signalling pathways. Both regulatory and metabolic pathways are mathematically described by ordinary differential equations and a uniform approach is used for describing the reaction kinetics. The modelling strategy allows for a (i) fast and reliable integration of various network layers to large-scale networks and (ii) fully-automated code-generation needed for simulation/parameter estimation based on time-series '-omics' data and high-performance grid computing. The

approach not only allows for predicting the effect of an external compound on intracellular concentrations, but also for predicting toxic effects triggered by inter-individual expression levels. INSIL developed and uses parameterised, compartmentalised dynamic mammalian cell line models. A kinetic metabolic HepG2 mammalian cell line model will serve as a starting *in silico* cell model within this project.

High-Performance Parameterization: Predictive long-term toxicity simulations require the integration of metabolic, regulatory, and detoxification pathways into large-scale, autonomous, and multi-scale network models. A critical assessment of network models reveals that (i) so far only relatively few intracellular compounds have been experimentally determined and mechanistically integrated in cellular network models and (ii) the identification/validation of large-scale cellular networks relies on using efficient solvers for simulating differential/algebraic systems and sophisticated parameter estimation routines, as well as on high-performance computing.

The need for high-performance computing stems from a large number of kinetic parameters that are usually not well known and which determine - in cooperation with the topology of the various network levels - the behaviour of intracellular compounds. Even with cutting-edge optimization strategies, billions of simulation runs have to be carried out for identifying the network dynamics and for estimating the reliability of the predictions as a function of the accuracy of the measurements. For embedding the network-oriented evaluation of '-omics' data into the modelling cycle, downscaling evaluation time through high performance grid computing is seen as an essential prerequisite. Today, grid computing networks built on modern multi-core processors allow for a highly scalable parallelisation of the time-critical simulation runs. In cooperation with the High-Performance Computing Centre HLRS (Stuttgart, Germany) the project partner INSIL runs large-scale cellular networks (1,000+ compounds) on up to 5,600 CPUs in parallel (NEC Nehalem grid computing cluster). Compared with a conventional desktop, the computing platform allows for network parameterization and network validation within weeks rather than years.

Modelling cellular and tissue characteristics: In liver, architecture and function are tightly linked. Consequently, one cannot expect that detoxification can be understood from experiments that consider only monolayers that largely neglect cell-cell communication. On the other hand, experiments in sandwich culture and organoids are more challenging than experiments in monolayer cultures, which is why mathematical models will be used to accompany and guide experiments in these systems. The moderate cell population sizes in the *in vitro* systems permit the use of agent-based mathematical models in which each cell is represented as an individual agent. The fundamental advantages of this model type are

permitting the mimicking of intracellular control and metabolism within each individual cell, and allowing for simulations at a one-cell-thick spatial resolution (*Figure 4.32*).

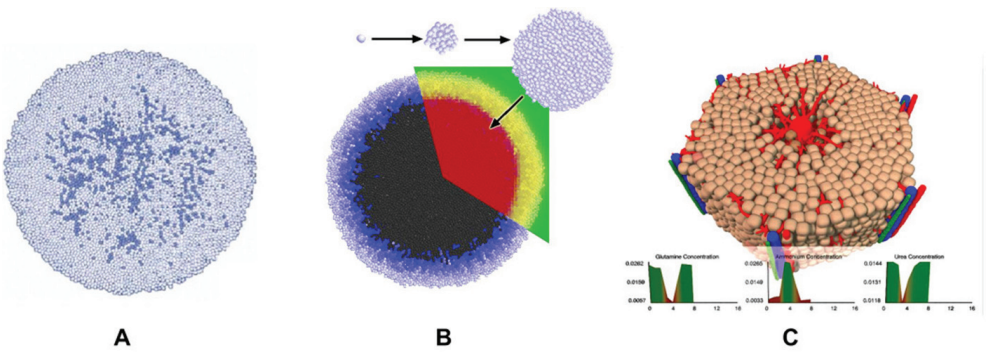

Figure 4.32 *(A) A simulated one-cell-thick compact monolayer in an agent-based lattice-free model. The dark blue regions denote cells undergoing apoptosis. (B) A simulated growth scenario of a multi-cellular spheroid. The large picture shows a cross section. Green, yellow and red colour denote large, intermediate and low glucose concentration, light blue the proliferating rim, dark blue quiescent cells and black the necrotic core (Drasdo and Höhme, 2005). (C) shows a snapshot from a glutamine, ammonia and urea concentration during liver lobule regeneration after intoxication by* CCl_4 *(Höhme et al., 2010).*

Experiments and model simulation results will be compared based on statistical measures that will be extracted by image processing and analysis from cell labelling experiments and experimentally determined distributions of the considered chemical agents (*Rohrschneider et al., 2007*).

Finally, as outlined below, by combining mathematical models that were calibrated in monolayers, sandwich cultures and organoids with architecture parameters obtained from human liver material **NOTOX** partner “Institut National de Recherche en Informatique et en Automatique” will be able to predict possible toxic effects in human liver and thereby contribute to bridging the gap between *in vitro* experiments and human.

Spatial-temporal modelling of tissue toxicity and functional consequences:

Recently, members of the **NOTOX** consortium have demonstrated that tissue toxicity and regeneration can be simulated in spatial-temporal mathematical models (*Höhme et al., 2010*). These models are based on tissue reconstruction from confocal laser scans (*Figure 4.33*) and experimentally determined process parameters, which initially have to be obtained from the *in vivo* situation. Based on this data certain aspects of toxicity to hepatotoxic compounds,

such as CCl_4 could be predicted with good precision (*Figure 4.34*). Examples are the spatial-temporal patterns of cell death and destruction of the liver microarchitecture (*Figure 4.35*) but also functional parameters, such as reduced ammonia detoxification as a consequence of application of hepatotoxic compounds. It should be considered, that this type of mathematical modelling will help reducing animal experiments. However, it may be a valuable tool for better and mechanisms based dose-extrapolations and for extrapolations between compounds that have a similar mechanism of action.

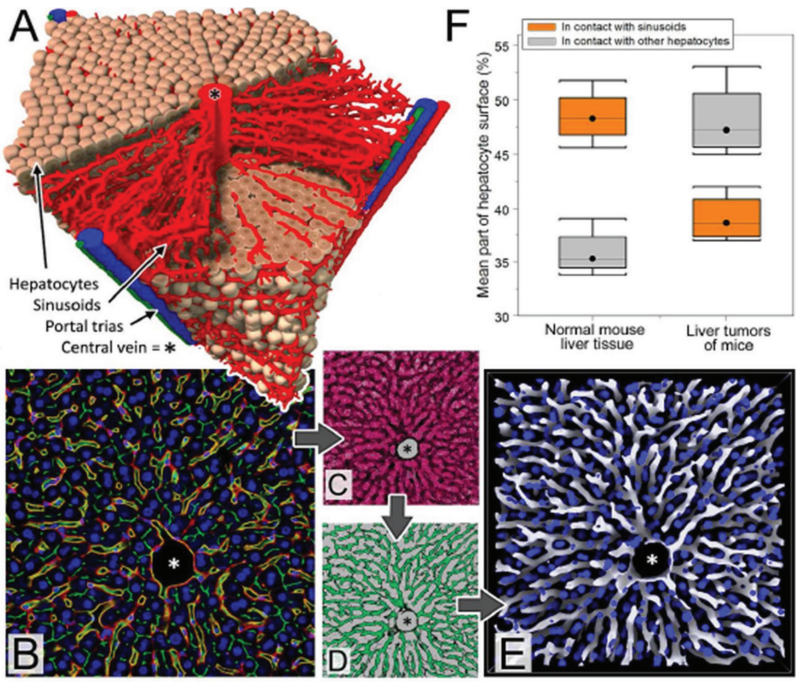

Figure 4.33 *Possibilities of mathematical spatial-temporal models to simulate aspects of liver toxicity. A. Liver lobule reconstructed from experimental data by the image processing chain B-E and successive image analysis. Such reconstructed, abstracted tissues serve as initial state for spatial-temporal mathematical modelling. B. Typical image obtained by confocal microscopy after adaptive histogram equalization filtering. Blue: DAPI (hepatocyte nuclei), yellow: ICAM+DPPIV (sinusoids), red: ICAM, green: DPPIV. C. Effect of generalised erosion filtering (all red pixels are removed). D. Effect of generalised dilatation filtering (all green pixels are added). E. Result of image processing chain in 3D. Blue: Hepatocyte nuclei, white: sinusoids. Note the complex architecture that links the periportal zone to the central vein in the middle of the lobule. F. Fraction of surface area of hepatocytes in contact with sinusoids (orange) and other hepatocytes (grey) in normal liver tissue and hepatocellular carcinomas of mice (from: Höhme et al., 2010).*

According to the state-of-the-art, spatial temporal models are available for simulations of acute liver toxicity and regeneration (*Höhme et al., 2010*). Progress beyond state-of-the-art will be achieved in **NOTOX** by exploiting the technique of spatial-temporal modelling also for long-term exposures with low dosages, by improving the possibilities of extrapolation to low dose levels and by improving the link between structural and functional (such as detoxification, protein synthesis, etc) aspects of liver toxicity.

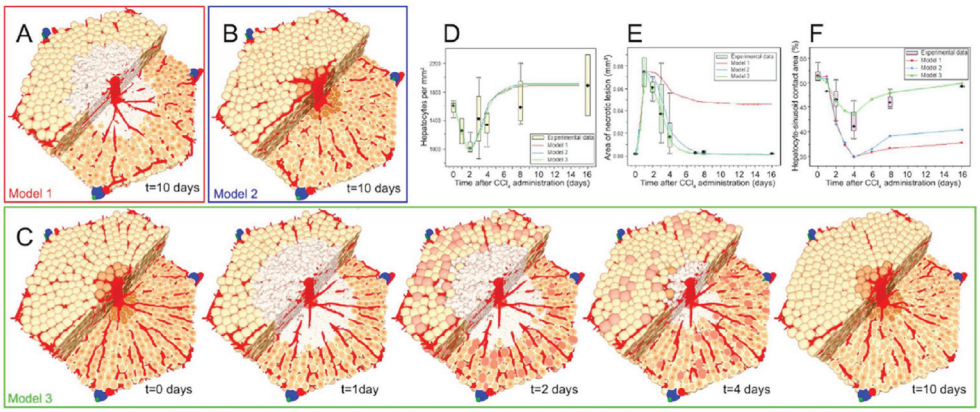

Figure 4.34 *Spatial-temporal model of liver toxicity caused by a hepatotoxic model compound that induces pericentral liver necrosis (CCl_4). Such models allow prediction of the alteration of tissue architecture as well as functional aspects. A goal of* **NOTOX** *is to extend spatial temporal modelling to long-term exposure and chronic toxicity (data source: Höhme et al., 2010).*

The systems biology challenge of combining heterogeneous data sources pursuing multiple goals at different points in time: The technical challenge is to somehow combine data sources as diverse as the scientific literature, DNA microarrays, high-throughput screens, modelling results and 3D structural information as derived within **NOTOX** and especially legacy animal toxicology studies to make usable and reliable predictive systems.

Modelling requires data, and models of biological systems need a great diversity of data from many different experiments. One must adopt innovative solutions that can cope with missing data to make predictive models. Better systems for data integration might just overcome some of the perceived problems of '-omics' technologies, for example microarrays or proteomics. Microarrays, for example, when used in isolation, suffer from problems of disease heterogeneity, limited sample availability and experimental variation that can lead to erroneous results or ineffective biomarker candidates. They, like many '-omics'-technologies, also suffer from an

'everything and nothing phenomenon' – providing too many data points to be understood; the answer to the question is probably there, but it is difficult to see through a fog of data. Almost anybody with experience advocates the use of a modicum of prior knowledge to make full use of these and other similar technologies (e.g. *Russo et al., 2003; Ein-Dor et al., 2006*). If one wishes, for instance, to use microarrays to study the molecular basis of response to a toxic chemical, then incorporating prior knowledge in the form of protein-chemical interactions can provide mechanistic suggestions very quickly (e.g. *Figure 4.35*). Simple integration of data can thus prove very rewarding. Combining '-omics' datasets both with each other and the abundance of prior knowledge from the literature or generated inside companies will clearly make them more effective in delivering their promises.

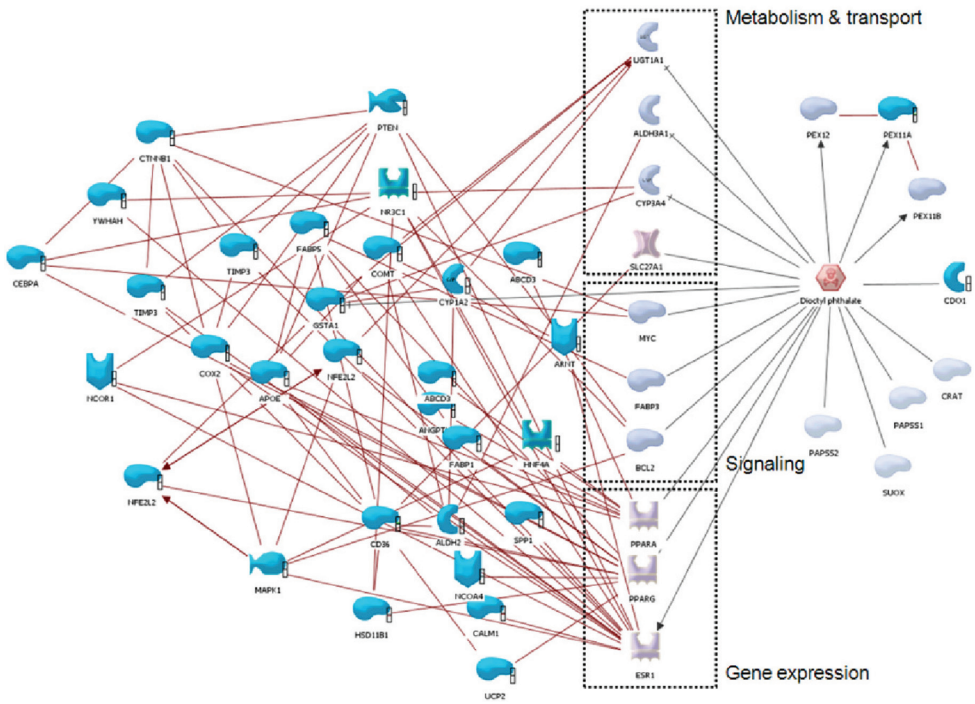

Figure 4.35 *Relating gene expression data to chemistry via protein-chemical interactions. Selection of genes affected in rat liver affected upon treatment by diethylhexyl phthalate. Protein-chemical interactions extracted from the literature show that the chemical likely induces changes in gene levels by first binding to a nuclear hormone receptor (PPARα/γ or ESR1), which is, in turn, responsible for turning the genes on or off in the living system. Classes of proteins/genes interacting with the chemical and other genes in the microarray study are boxed. Microarray datasets are from the EDGE (Environment, Drugs and Gene Expression) database; protein chemical interactions and data integration/visualization was performed using ToxWiz (Cambridge Cell Networks Ltd., UK).*

4.6.5 Innovation

The overall strategy of the work plan is presented in *Figure 4.36*. Hepatocyte and liver organotypic cultures will be carried out in relevant technologies. 3D membrane cultures, spheroid cultures and sandwich cultures of liver cells will be operated in parallel. Initially used liver cells include HepaRG and primary hepatocytes. Mouse liver organoids will be supplied by "The Netherlands Cancer Institute" as available. These will be cultivated and monitored with or without exposure to selected test compounds. Test compounds will be selected in cooperation with ToxBank (project of the **SEURAT-1** Research Initiative, see following chapter). These test compounds will be applied in repeated doses over long-term at different concentrations. Viability, CYP and other relevant enzyme activities will be monitored as well as extracellular –omics data will be collected at regular intervals. Dynamic experiments with high frequency measurement of intracellular metabolites after ^{13}C labelling will assist configuring and parameterising mathematical models. Intracellular –omics data, particularly transcriptomic, and epigenetic data, will be measured at selected time points. 3D structures of tissues will be studied using both, light and electron microscopy and tomography.

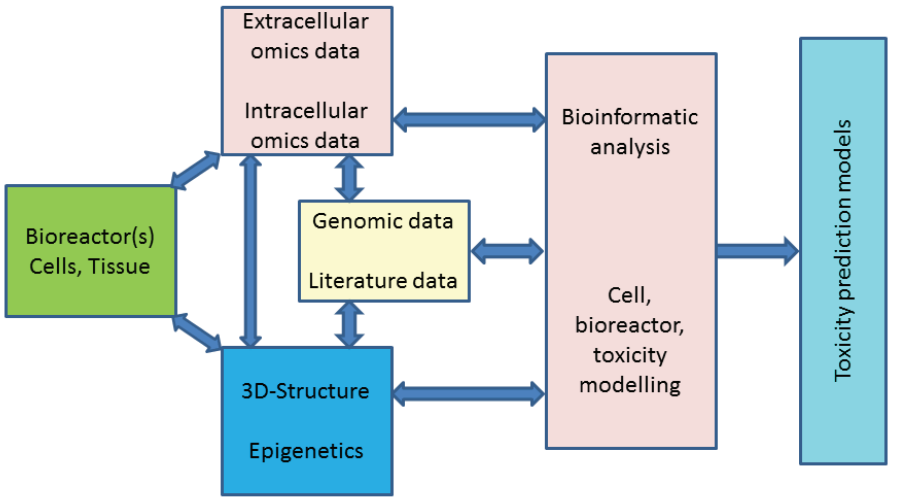

Figure 4.36 *Toxicity modelling and prediction.*

Transcriptome, proteome, metabolome and fluxom data will be integrated in large-scale metabolic and regulatory network models. Parameter estimation will be carried out in close cooperation among the involved partners. 3D structural data will first be treated with new computational techniques and used to guide cell and tissue modelling. These models will also include relevant data from the network models. Gemone, epigenome, transcription,

metabolome, fluxome and proteome data will be incorporated in bioinformatic models and compared across species using literature data and curated data from databases. Exchange of data with other projects of the **SEURAT-1** Research Initiative will primarily be made using databases and data management structures supplied by **NOTOX** in collaboration with the data warehouse in ToxBank. A quantitative understanding of toxicity response pathways and networks at the molecular and macromolecular level with computational modelling efforts will help indicate toxicity response triggers. Human cell based systems that reflect physiological conditions (organotypic 3D cultures) should ensure the relevance of the system and therefore improve the predictive power.

Most important innovative aspects of **NOTOX** are:

- 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 light and electron microscopy and tomography methods and model such effects for prediction of human toxicity
- Create large-scale computational models supported by databases for prediction of human long-term toxicity based on *in vitro* data

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

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4.7.1 Introduction

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. The following activities will be carried out:

a) Establishment of a dedicated web-based data warehouse

The **ToxBank** Data Warehouse (TBDW) will establish a centralised compilation of data for systemic toxicity. Data generated under the research program and additional public data will be uploaded and integrated whenever possible into computerised models capable of predicting repeated-dose toxicity.

b) Establishment of a database of test compounds

The **ToxBank** Gold Compound Database (TBGCD) will provide a high-quality information resource servicing the selection and use of test compounds. Chemicals in this database will be supported by *in vivo* and *in vitro* repeated-dose toxicity data, property data and, whenever available, human adverse event and epidemiological data. Selected test compounds for training or validation, and standard operating procedures (SOPs) for data quality control, processing and analyses will be provided.

c) Establishment of a repository for the selected test compounds

The **ToxBank** Chemical Repository (TBCR) will ensure the availability of test compounds to program researchers accompanied by sample preparation, handling and analytical quality control procedures.

d) Setting up of a cell and tissue banking information resource for in vitro toxicity testing

The **ToxBank** BioBank (TBBB) will establish a banking information resource for access to qualified cells, cell lines (including stem cells and stem cell lines) and tissues and reference materials to be used for *in vitro* predictive toxicology research and testing activities.

4.7.2 Objectives

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

- Collaboratively establish the requirements for data management and modelling, chemical compounds, and cell and tissue biological reagents for systemic toxicity research methods across all projects of the **SEURAT-1** Research Initiative
- Establish a Data Warehouse of Linked Resources which hosts and provides access to a centralised compilation of all data from the **SEURAT-1** Research Initiative (both experimental and processed data), public data from high-quality repeated-dose *in vivo* and *in vitro* studies, together with ontologies and computer models generated from the data
- Develop web-based interfaces for linking and loading raw and processed data into the Data Warehouse infrastructure as well as accessing the data and modelling results, including methods for searching, visualisation, property calculation and data mining
- Specify standardised requirements for annotation and submission of "-omics" and functional data produced by the projects of the **SEURAT-1** Research Initiative 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.)
- Select 'gold standard' test compounds ('Gold Compounds') having high-quality data and providing chemical and biological diversity across a range of repeated-dose toxicity endpoints
- Create a TBGCD for the import, curation, acceptance and storage of quality data related to Gold Compounds

➠ Support education and ensure compliance within the **SEURAT-1** Research Initiative with procedures and data submission requirements and obligations to fulfil an Integrated Data Analysis strategy across the complete program

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

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

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

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

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

➠ Develop the program capacity for establishing quality and reliability goals in methods

➠ Develop the program capacity for the reliable estimation of uncertainty in predictive models

➠ Establish a sustainable infrastructure of resources supporting and servicing all current requirements for systemic toxicology Research and Development that is extensible to future requirements for validation and risk assessment acceptance for industrial and regulatory needs

4.7.3 State of the Art

One critical goal of the **SEURAT-1** Research Initiative is to create integrated data analysis capability across all program projects so as to avoid fragmented outcomes. Unfortunately current cultures and their work practices are often ineffective in achieving such higher–level missions. Researchers can be reluctant to share data, especially when research activities are running prior to publication.

The **SEURAT-1** Research Initiative represents the first step of the 'trias' of technological development, validation and regulatory acceptance. To avoid developments which will not be

carried further, it is important to address already at the design stage of R&D activities future validation and regulatory acceptance needs. The most important aspect is the clear definition of methods, normally as Standard Operating Procedures (SOPs). In the past, the validation of methods has often been impaired by ill-defined, untransferable protocols. The request was made that validation studies should be carried out under quality assurance schemes such as Good Laboratory Practice (*OECD, 2004*). Most importantly, a set of guidance called Good Cell Culture Practice (GCCP) was developed (*Hartung et al., 2002; Coecke et al., 2005*) to improve this aspect and which will be critical for the introduction of new and potentially more relevant assays. GCCP has already been adopted by key scientific organisations (e.g., European Society for Animal Cell Technology, MRC, UK) and referenced in new WHO recommendations on cell culture practice for manufacturing using cell-based production systems. The definition of a test method became formally an accepted module of test validation in 2004 (*Hartung et al., 2004*). It was accepted also in the respective OECD guidance (*OECD, 2005*). However, for validation purposes different steps need to be taken: the point of reference needs to be defined; criteria were elucidated in an ECVAM workshop (*Hoffmann et al., 2008*) as were the principles of weighing evidence (*Balls et al., 2006*). **ToxBank** will implement support for partners in the **SEURAT-1** Research Initiative to access and use SOPs for data, compounds and biomaterials.

Several projects funded by the European Commission (e.g., ReProTect *(2011)*, Predictomics *(2011)*, carcinoGENOMICS (*Vinken et al., 2008*), Sens-it-iv *(2011)*, *OSIRIS (2011)*, CAESAR *(2011)*, ESNATS *(2011)*, LIINTOP (*Gómez-Lechón et al., 2010*), ACuteTox *(2011)* and ECVAM (*Kirkland et al., 2008*) have needed to address the issue of compound selection for use in their experimental developments and **ToxBank** will draw on their experiences. Compound sets have often been relatively small in size and, because of the specific focus of a given project, not generally coordinated across multiple projects. Experiences in successful development of alternative methods have, however, shown the need for a strong coordinating action and an integrated data management based on a carefully selected set of reference compounds (*Schenk et al., 2010*).

Currently within EC funded research initiatives, the fragmentation of compound resources, selection and application activities results in the lack of standardised high-quality compound sets with integrated use across alternative testing projects. Furthermore, the absence of a sustained controlled supply of test compounds hampers progress in the ultimate strategic goal of industry and regulatory acceptance of a particular new alternative method.

CAESAR found that the lists of chemicals used in toxicity laboratory experiments reported within recently published literature contained errors for ca. 10% of the compounds (*Zhao et al., 2008*). Even collections of chemicals claimed to be ‘gold standards’ contained serious errors. The most typical errors were in the definition of the chemical structure (positions of the substituents, definition of isomers, and characterization of mixtures). Another observed error was the lack of recognition that chemicals may undergo degradation, transformation

or hydrolysis within the experimental conditions. Several chemicals for instance were found to hydrolyse within a few hours, while the reported bio-concentration had been referred to the parent compound, even though it was no longer present in the experimental conditions of assays running over days or weeks. Together, these errors imply an incorrect assumption of the chemical identities and properties of the test compounds in assays, which should be removed through pre-emptive analysis. The EU-project OSIRIS (2011) reached similar conclusions stressing the need for a quality check of test chemical data that is higher than typical current practices.

Extensive international haematopoietic cell and tissue banking networks are now well established for clinical application, and there are also well developed general networks of microbial collections which have incorporated collections of human and animal cell lines used for broad research applications. However, there is an important need within the **SEURAT-1** Research Initiative for a coordinated activity to enable biologists, engineers and toxicologists to accelerate the development of more relevant *in vitro* assays through access to appropriately qualified sources of tissues and cell lines. Promoting access to such resources must also be matched by the establishment of quality standards that will promote the quality and standardisation of toxicology research and prevent research time being wasted on poor-quality materials, as prescribed in GCCP.

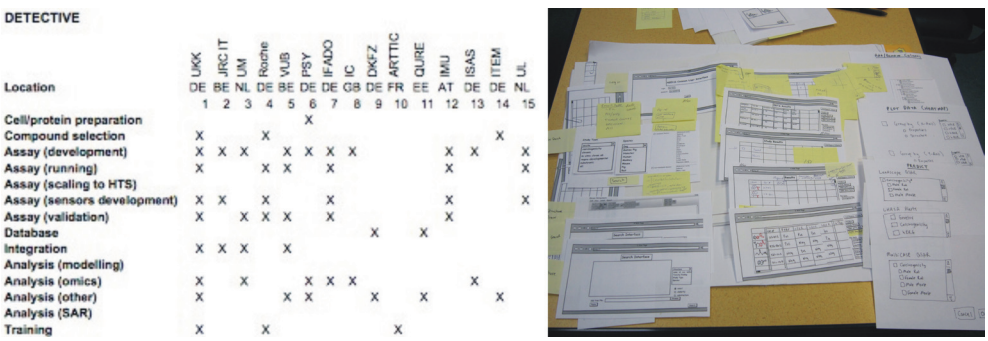
Human stem cell lines clearly have the potential to deliver human *in vitro* models for any tissue of the body and there are initiatives within individual countries and states (e.g. Netherlands (*ASAT, 2011*), UK (*SC4SM, 2011*), USA (*CIRM, 2011*) but as yet, no formally coordinated activity to ensure that panels of appropriate genotypes of stem cell lines qualified for their suitability and utility in toxicology studies are available for open use by researchers, industry and testing companies. There are few active resource centres distributing human stem cell lines internationally, and the lead groups in this area are the *UK Stem Cell Bank (2011)* established by **ToxBank** partner NIBSC, *WiCell (2011)* and the *US NSCB (2011)*. Internationally the lead coordinating activity for the supply of human stem cell lines for research and clinical application is the International Stem Cell Banking Initiative, led by the UK Stem Cell Bank and funded by a consortium of national stem cell research funding bodies. Control preparations and reference materials for test cells and tissues will also be important considerations with special regard to measurable phenotype, cross-contamination of cell lines and stability *in vitro* (*Stacey, 2007*).

4.7.4 Approach

Infrastructure and Services

Requirements analysis will be a key initial and ongoing activity in the **ToxBank** project which has a mission of servicing the needs of all researchers on the **SEURAT-1** Research Initiative (*Figure 4.37*). Significant communications and discussion will be needed with users on the different projects so as to capture the significant Use Cases and community standards in need

of servicing, including those for data submission and analysis, and test chemical and biological sample requirements. Site visits with program partners will be carried out to determine how **ToxBank** services can support users in all steps of their research including planning, retrieving existing data, selecting and handling compounds and biomaterials, accessing SOPs, ordering supplies, uploading and analyzing data subsequent to completion of experiments, and model building and reporting.



DETECTIVE

Location	UKK DE 1	JRC IT BE 2	UM NL 3	Roche DE 4	VUB BE 5	PSY DE 6	IF-ADO DE 7	IC GB 8	DKFZ DE 9	ARTTIC FR 10	QURE EE 11	IMU AT 12	ISAS DE 13	ITEM DE 14	UL NL 15
Cell/protein preparation						X									
Compound selection	X			X										X	
Assay (development)	X	X	X		X	X	X	X				X	X		X
Assay (running)	X			X	X		X					X			X
Assay (scaling to HTS)															
Assay (sensors development)	X	X		X			X					X			X
Assay (validation)	X		X	X	X		X					X			
Database									X		X				
Integration	X	X	X		X										
Analysis (modelling)															
Analysis (omics)	X		X			X	X	X					X		
Analysis (other)	X				X	X			X		X			X	
Analysis (SAR)															
Training	X			X						X					

Figure 4.37 *Starting point of the requirements analysis: identification of potential needs of* ***SEURAT-1*** *projects.*

Best quality data sharing, management and analysis procedures and policies including data uploading and quality measures will be implemented by **ToxBank** in compliance with the integrated data management guidance specified by the **SEURAT-1** Cluster Agreement. **ToxBank** will develop an interoperable platform suitable for adoption by pan-European and multinational organisations, providing interfaces for partners and additional suppliers to plug-in their resources to the platform. **ToxBank** will also develop a strategy to limit data sharing barriers, which may include easier interfaces (to avoid additional work for data submission), incentives for data sharing (e.g., automated creation of integrated datasets), and differentiated data access policies (e.g., access-based services for confidential data, enabling both time- and context-dependent restriction and access enlargement by data owners).

We propose to develop a “Linked Resource” approach which can be used to link information resources developed locally by projects of the **SEURAT-1** Research Initiative through standardised Application Programming Interfaces (APIs, *Figure 4.38*). As an example, functional and “-omics” data from the DETECTIVE project could be processed locally on a day-to-day basis by its own data management and analysis team. Through pre-agreement on data services supported by OpenTox APIs (*Hardy et al., 2010*) or any needed extensions, we can deliver data from **ToxBank** web services to the DETECTIVE analysis e.g., *in vivo* data related to the endpoint under analysis, test gold compound data, and linked data from other

projects which would be made available either through user web queries from the **ToxBank** portal or directly via APIs to local systems. The DETECTIVE analyst could process raw data locally and link in processed data resources to be used by others in their analysis through the APIs supported by **ToxBank**: this data could be in different representations, granularity and formats, but a chain of links would allow linking back to lower levels of source data for subsequent access. Data labelling including for quality or granularity from local curation could be linked to **ToxBank** authorisation and authentication services and access policies, which would hence define in a controlled manner the data release, format and timing to any program user, partner, project, program or the world. The technical details of the approach to data management and exchange between all projects of the **SEURAT-1** Research Initiative and **ToxBank** will be formulated during the requirements analysis and design phases carried out in 2011. The proposed Linked Resource approach is not restricted to data resources alone but can also be applied to other types of resources of relevance to integrated analysis such as algorithms, validation, models, applicability domain, and reporting, as has been developed under the EU project *OpenTox (2011)* and can be both reused and extended as needed. This approach could also be used to link assays to genes, pathways and biological effects through ontology services.

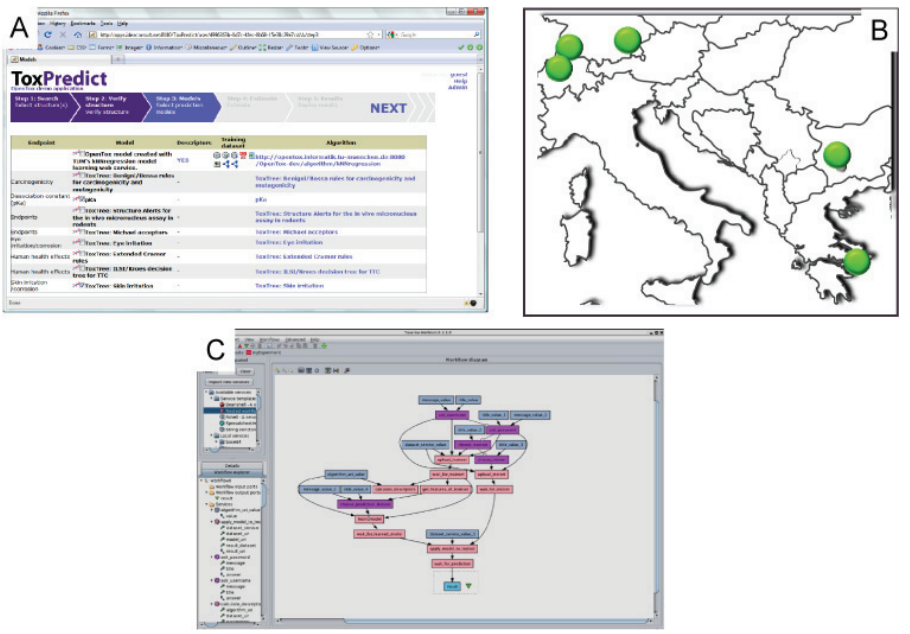


Figure 4.38 *Linked Resource approach in action with OpenTox. A: Simple building of predictive toxicology applications based on well-established methods and databases. B: Distributed applications, integrating wide range of data, models and prediction methods. C: Integration into workflow systems for computational biology.*

ToxBank Resources

Data Warehouse (TBDW): The web-based TBDW will house and provide access to the centralised compilation of data related to repeated-dose toxicity and interfaces to distributed resources created within the **SEURAT-1** Research Initiative or elsewhere (*Figure 4.39*). The TBDW will support the selection of the model compounds for the TBGCD and the data management and analysis efforts for all projects of the **SEURAT-1** Research Initiative. The TBDW will provide access to relevant public data as well as raw and processed data from all program projects. Web-based interfaces will be developed to both load and link data resources. The TBDW will be designed so as to support standards development and interoperability for toxicity resources through the use of ontologies, ToxML schema (*Leadscope, 2011; Lhasa Limited, 2011*), and OpenTox-compliant APIs for system components, thus enabling the integration of resources and tools both from within and outside the project. The incorporation of vocabularies and ontologies will enable the reliable and standardised description and integration of data and models, and will support complex inferencing queries and integrated analysis.

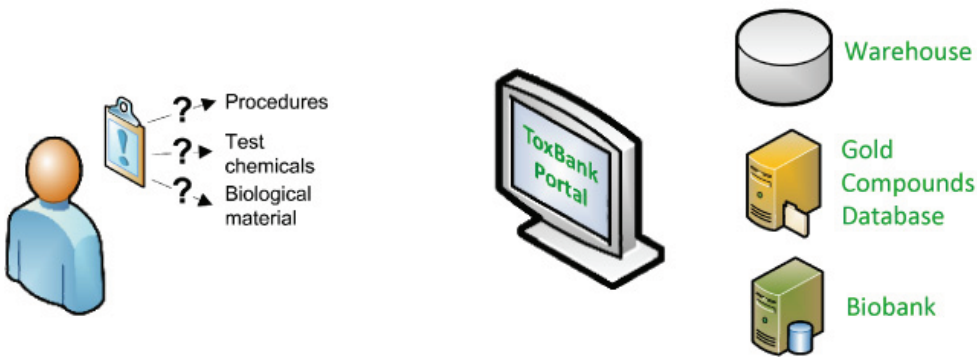


Figure 4.39 *Overview about the* **ToxBank** *Resources. The user examines existing data and models related to the toxicology end-point studied.*

The TBDW will import existing and public systemic toxicity data into an internal representation based on ToxML data schema (*Leadscope, 2011; Lhasa Limited, 2011*). To support the representation and data management of new *in vitro* assays developed within the program, collaborative work with assay developers will be required to extend vocabulary and data schema to the new assays. It is expected that strong collaboration opportunities with all projects will also exist in activity for the standardised description of '-omics' data, protocols, samples, mechanisms and systems biology models. Existing ontologies will be reviewed with a working group of developers in the **SEURAT-1** Research Initiative and users and external experts to consider reuse of existing work such as available from neighbouring ontologies.

The TBDW will support the management of chemical information in a chemical data mart

accompanied by an update to OpenTox-compliant API standards. The system will include state-of-the-art handling of chemicals including error checking, aromatic systems, tautomers, and stereochemistry.

The TBDW will also implement a data infrastructure for the storage of biological data such as "-omics" data used by the DETECTIVE and NOTOX projects (*Figure 4.40)*. The infrastructure will store and track experimental procedures, protocols, measurements and results, thus allowing cross-experimental data analysis. It will also carry out data transformations and data mining, and provide a web interface for uploading and viewing data, Excel file upload, visual depiction of dose-response curves, and meta-analysis. Each experimental, measurement or data analysis step and all computations (e.g. calculation of EC_{50} values, normalisation of array data, data mining procedures) will be documented with standardised protocols. This will allow users to trace data analysis results back to their origins (e.g. experimental settings, protocols). Services will handle data and user security including controlled policy access to data as well as providing appropriate backup and archiving services.

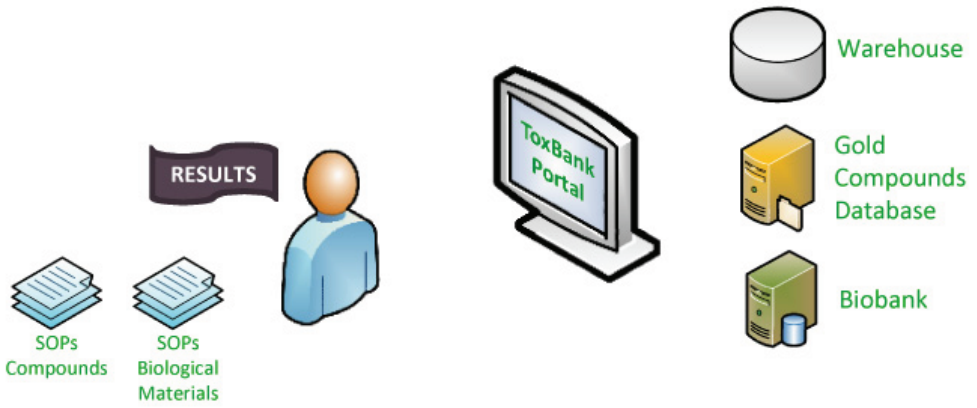


Figure 4.40 *User uploads results using Data Import Facility.*

Gold Compounds Database (TBGCD): Since a main goal of the **SEURAT-1** Research Initiative is to create *in vitro* assays for the prediction of systemic toxicity, the primary purpose of the compound standards in this project is to serve as tools for the development and testing of these assays. In this regard, the compound set design will need to achieve chemical and biological diversity goals probing mechanisms of action by the specific *in vitro* assays under development. Our compound selection and 'gold standard' set will address these requirements through the evaluation of existing knowledge and interactions with experimentalists on the other projects of the **SEURAT-1** Research Initiative.

We will create a quality-controlled curated cheminformatics TBGCD database for 'gold standard' reference compounds that can be used in the training and validation of alternative

testing *in vitro* assays and *in silico* models of systemic toxicity. Gold Compound selection criteria and standardised curation and operating procedures will be established early in the project to support the earliest possible creation of the database and its use in decision making on R&D project and assay design across the **SEURAT-1** Research Initiative (*Figure 4.41*). Our goal will be to provide a chemical diversity of sufficient structures in each category relevant for endpoints studied in the **SEURAT-1** projects.

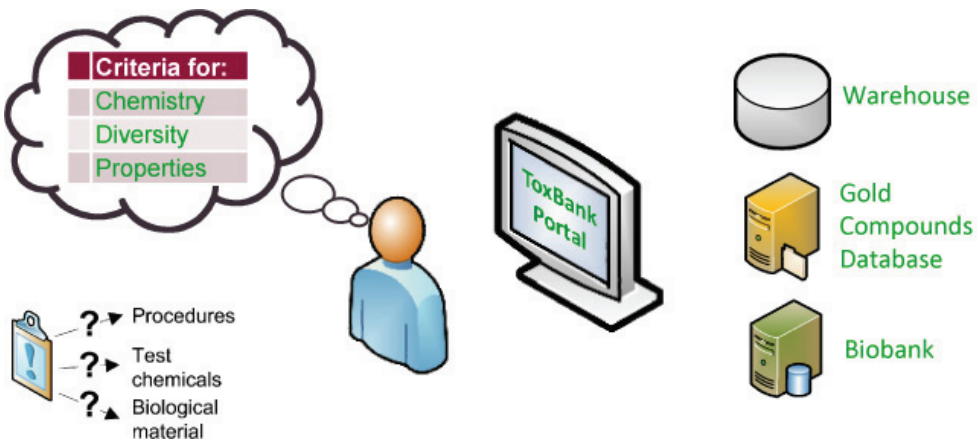


Figure 4.41 *User queries Gold Compounds Database.*

Identification of compounds that possess agreed biological characterization will be carried out as an objective process using the data mining infrastructure of the TBDW. Selection of compounds based on diversity of chemical structure will be accomplished using toxicological mechanism of action, standard Tanimoto criteria of chemical structure similarity, and physico-chemical properties that are consistent with ease of storage and handling in high throughput *in vitro* assays. The TBGCD will be implemented in compliance with TBDW designs and architecture. Workflows will be established for data evaluation, curation and acceptance, including the capture of quality indicators and metadata into TBGCD fields. Data mining tools for test compounds selection will be created.

Chemical Repository (TBCR): The TBCR will manage the characterization, handling procedures and distribution of test compounds for the program. We will prospectively guide testing procedures with all the necessary and correct information related to the used test chemicals. In some cases, the presence of trace compounds may represent toxicity problems and we will evaluate the possible occurrence of highly toxic impurities (e.g. nitrosoamines, dioxins, aromatic amines) on the basis of the chemical structure of the parent compound. We will identify the possible occurrence of stereoisomers (e.g. enantiomers, diastereoisomers,

double bond isomers), and define their nature for all test compounds. For all compounds we will evaluate stability under the experimental conditions used within the **SEURAT-1** Research Initiative. Furthermore, in case of potential problems, as predicted by *in silico* methods, or on the basis of inputs from the projects of the **SEURAT-1** Research Initiative, we will measure the stability of the chemical. For this, analytical methods will be developed, using mass spectrometry and chromatography, to carry out experiments to measure stability and eventual degradation.

Software tools will be used to calculate test compound physico-chemical and biodegradation properties. Occurrence of tautomers will be evaluated to provide useful information to both laboratories working on *in silico* models and experimental assays. These properties will be further improved on the basis of the experimental data obtained at the repository analysis facilities.

We will predict whether the test chemicals, used within the **SEURAT-1** Research Initiative, will bind in a non-specific way to proteins present in medium such as serum. For this we will evaluate the *in silico* models used for pharmaceutical compounds. For chemicals with missing data or out of model applicability domain or showing anomalous results in experiments conducted within the **SEURAT-1** Research Initiative, new binding property measurements will be carried out.

To foster standardisation of the newly developed methods, standard procedures for handling the chemicals will be optimised and distributed to program partners. Complementarily, a standard template for the preparation of test protocols as SOPs will be set up, also according to regulatory requirements, including what is needed for the REACH registration of the chemicals.

BioBank (TBBB): The TBBB will involve the development of an information resource on biological sample banks which can service the repeated-dose toxicology requirements of the other projects with appropriate cells, tissues and cell lines (hereinafter 'Biomaterials') for use in assays including specialised control materials (*Figure 4.42*). Stem cells play a particularly important role in the **SEURAT-1** Research Initiative: in the functional differentiation goals of *SCR&Tox* to produce cells of toxicological relevance, e.g. to support development of hepatic cell assays for human liver toxicity assessment or to reproducibly generate organ-specific cells and tissues for the organ simulating devices to be developed in *HeMiBio*. Strategies to achieve differentiated organ-specific cells by sequential exposure of stem cells to a series of growth factors reflecting *in vivo* embryogenesis, will require attention to the availability and quality control of stable cell line samples. The TBBB will primarily focus on materials of human origin although important resources of non-human cells and tissues will be addressed where human-derived alternatives are not available or are still under development.

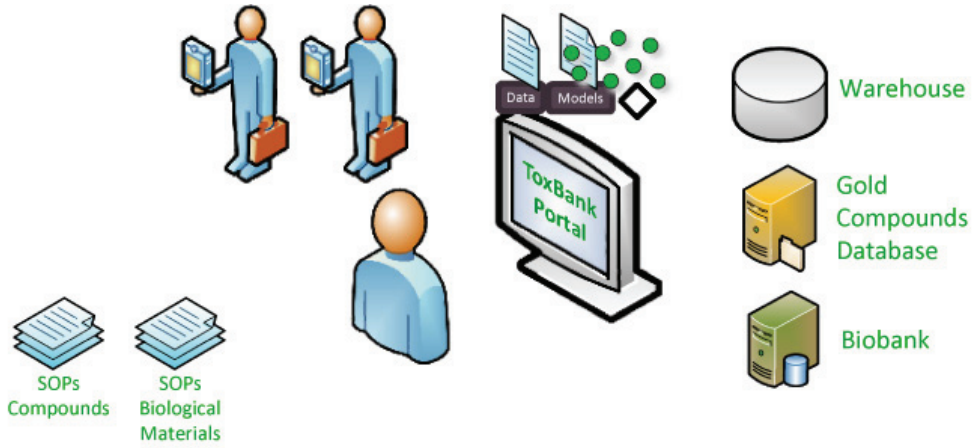


Figure 4.42 *User orders compounds and biological materials.*

We will first review the general Biomaterial supply resources available and identify those with special relevance to *in vitro* systemic toxicology and the detailed user requirements identified in the requirements analysis. Based on information provided by suppliers and users, an evaluation and recruitment process will be set up to produce a web-based registry of suppliers. An overall framework for managing the procurement and supply of Biomaterials will be established that will be coordinated as a consensus with suppliers, user groups and other stakeholders for the provision of materials of acceptable quality. Key scientific direction for the establishment of generic criteria will be established through an expert scientific user group and questionnaires to the general user population who will also be engaged to identify a panel of key cell lines and tissues for toxicology work (e.g. number and composition of genotypes required for toxicity safety testing). This framework will form the basis for the final operational system of the Biomaterial supplier network. Detailed scientific and safety quality criteria and protocols will be required and established for each type of Biomaterial.

An early priority will be to define general quality and regulatory criteria for establishment, storage, testing and dissemination of Biomaterials. These will be coordinated with the supplier network to establish consensus best practice which meets regulatory requirements for procurement and transfer of Biomaterials to resource centres. These criteria will be used to begin the formulation of a suppliers' charter for provision of Biomaterials.

Testing procedures will be developed to include those of significance for safety of laboratory workers as well as quality of scientific investigation based on the Biomaterials. Procedures will be developed for pre-supply scrutiny of recipients with respect to their local regulation and law; this may include an independent scientific evaluation to establish value of proposed uses of Biomaterials. Traceability and provision of information on shipping requirements and regulations will also be a part of this activity to support the user community.

Having identified a supplier network, an evaluation process will be established to invite feedback from suppliers of tissues and primary cells, indicating how they meet the established quality, research and regulatory criteria. All suppliers will be listed with their authorised response to the evaluation process and any quality standards or regulations under which they are inspected or to which they propose compliance. Ongoing supply of Biomaterials under the proposed framework will require formation of a virtual network of suppliers signed up to the project charter encompassing operational, quality and safety standards.

Quality Control

The use of human biological substrates is prone to a whole range of variables and complications and errors relating to the substrate preparation. Mixups during processing of tissues or cells can lead to material being supplied under the wrong name, and in the case of cell lines the substrates can be replicated readily *in vitro* and passed to other workers which has led to serious concerns regarding the research funding wasted in R&D on cross-contaminated or switched cell lines. Cell lines are also prone to alteration in phenotype and genotype due to extensive passage *in vitro* leading to genetic drift. Dramatic alterations to the cell performance can also arise from persistent contamination with micro-organisms (*mycoplasma spp*., non-lytic viral infections). Accordingly sourcing of substrates and cell lines in particular require careful quality control and characterization, and centres supplying such material for R&D should be performing such quality control. Furthermore this quality discipline should be established in the user laboratories as the issues of material authenticity, purity and stability remain error prone in the general laboratory environment. **ToxBank** is therefore committed to providing a system by which toxicologists wishing to source human cell and tissue substrates for their research will be able to identify those centres providing quality-controlled materials with characterisation appropriate for use in toxicity research. These issues are even more important in the use of technically challenging culture such as human stem cell lines (both hESC and iPSC) where the preparation of stock for use in experimental work can take months and an error in selection of the correct cell line or provision of cross-contaminated or altered cell lines will result in significant waste of time and resources. Thus **ToxBank** biobanking operations not only have to ensure screening of suppliers for quality control but also will actively engage the user community, identify particular substrate systems (e.g., hepatocytes, cardiomiocytes) that can be derived from cell substrates on offer, but also particular genotypes (genetic background or number of panels of different genotypes) that will be required to deliver validated culture systems to meet industry standards and regulation.

The use of human stem cell lines of all types (adult, fetal, iPSC and embryonic) will bring a special focus on the above characterisation and quality issues, as they are particularly dynamic systems containing at any one time a mixture of undifferentiated stem cells and a background population of cells potentially in various different differentiated states. The balance

between these cell types is known to vary from one time point to another and differentiated populations can quite readily take over which are not desired for the particular purpose of *in vitro* modelling of human-differentiated cultures. Control of such unwanted differentiation is a major challenge for this area of work and **ToxBank**, through its advisory web pages and user selection mechanisms for appropriate suppliers, will actively promote good cell culture practice and good stem cell banking practice (*The International Stem Cell Banking Initiative, 2009*). In addition through the involvement of the UK Stem Cell Bank and other suppliers of stem cell lines coordinating with the International Stem Cell Banking Initiative and International Stem Cell initiative on characterisation, guidance on phenotypic and genotypic evaluation of human stem cell lines will be established for the user community of the **SEURAT-1** Research Initiative.

ToxBank Storyboard Vision

As shown in *Figure 4.43*, once assembled, the **ToxBank** resources of the Data Warehouse, Gold Compound Database, Compound Repository and Cell and Tissue Bank will support users in all steps of their research including planning, retrieving existing data, selecting compounds, biomaterials, accessing SOPs, ordering supplies, uploading and analyzing data subsequent to completion of experiments, and model building and reporting.

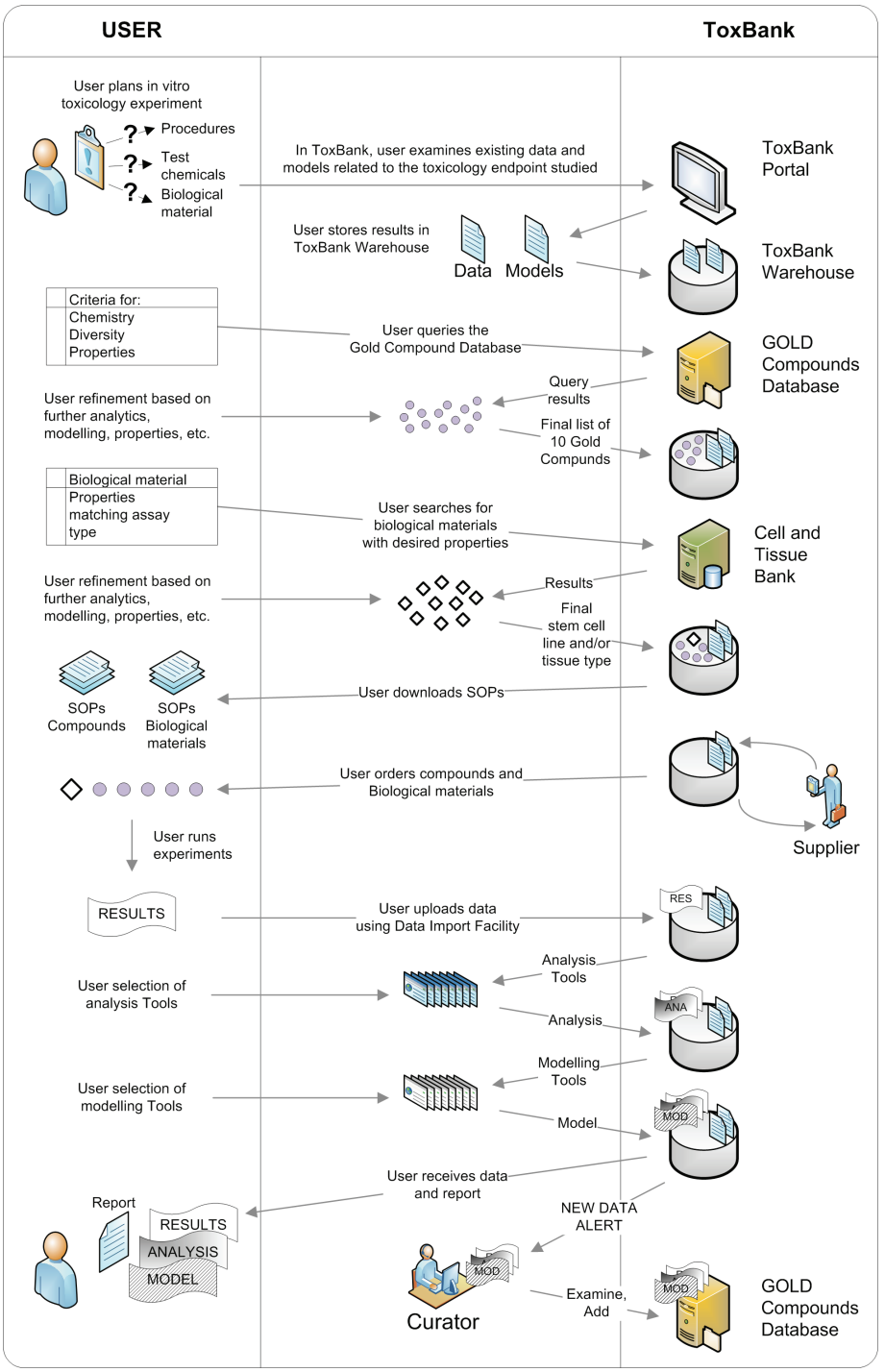


Figure 4.43 **ToxBank** *support of partners in the* **SEURAT-1** *Research Initiative.*

Sustainability

Based on project experiences and stakeholder interactions, a detailed service model and business plan will be created across all major **ToxBank** resources. The makeup of the **ToxBank** consortium reflects the importance of servicing users through the strong involvement of numerous organisations already working in the toxicity product and service area whose day-to-day operations reflect the primary business value of servicing customer needs. This unique business strength of the consortium should optimise the support functions for **ToxBank** resources and ensure service and business models that provide sustainability beyond the end of the project. **ToxBank**'s culture of service combined with the culture of research of the other projects will provide mutual overall strengthening of the program and achievement of its challenging long-term goals.

4.7.5 Innovation

The expected overall impact of the **SEURAT-1** Research Initiative is the development of human safety assessment strategies based on alternative method tests with higher predictive value and lower cost than traditional animal-based toxicity experiments. Such strategies aim to replace (replace component of '3Rs' principle) repeated-dose systemic toxicity testing historically carried out in animals.

The **ToxBank** project establishes critical infrastructure and services functions to all program projects providing a centralised and standardised set of data resources, compounds, and biological samples accompanied by standardised operating procedures and guidance. The positioning of **ToxBank** within the project structure of the **SEURAT-1** Research Initiative is illustrated in *Figure 4.44*. The establishment of such an infrastructure project aiming at the integration of EC research efforts among the project cluster partners from the beginning on is unique. The provision of quality sources of compounds, cells and tissues for research will promote novel human cell based assays that will facilitate a more accurate evaluation of toxicity. These resources will ensure that the alternative *in vitro* assays developed by research activities of the programme are guided and supported from an early stage of design, to maximise their potential of reaching the pre-validation stage as defined by ECVAM, and eventual validation and regulatory acceptance such as required under the Cosmetics Directive and REACH. Thus, regulatory agencies are target beneficiaries for this infrastructure. REACH places a significant demand on all businesses operating in the European marketplace involved in the import and manufacture of products involving chemical entities; furthermore companies are required to address the '3Rs' principles and evaluate and potentially use and report on alternatives, wherever possible. Thus, industry is another major target stakeholder for our infrastructure as industry-standard resource facilities such as ToxBank are required for risk assessment activity. SMEs particularly will be challenged by REACH as they frequently do not

have in-house tools and knowledge resources for the assessment work. **ToxBank** also should have beneficial impact on Colipa and other organizations affected by the Cosmetics Directive which places a very strong legislative 3Rs requirement on consumer product companies.

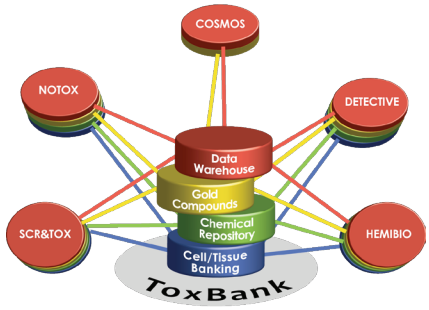


Figure 4.44 *Positioning of ToxBank within the* ***SEURAT-1*** *Research Initiative.*

The TBDW creates a centralised repository for all data created during the research program of the **SEURAT-1** Research Initiative in addition to importation of relevant *in vitro*, *in vivo* and human data from other sources. Well-designed data import facilities will support ongoing data collection according to quality guidance. **ToxBank**'s use and further development of data exchange standards for describing systemic toxicity data will reduce errors and enable data integration from different laboratories, including data sources outside the **SEURAT-1** Research Initiative. This approach will enable the earlier sharing and analysis of data between program partners, so as to optimise insights, collaboration, and communications to support decision making with regards to promising discoveries or changes of direction to overcome obstacles. Furthermore the support of such an integrated data analysis will be critical to achieve the ultimate goal of predictive models that can be subsequently extended to validation, e.g., the combination of predictions from *in silico* and *in vitro* methods can be used to provide a combined weight of evidence and consensus models with greater predictive power than single methods used alone.

The **ToxBank** approach to interoperability will support a fruitful collaboration on the **SEURAT-1** Research Initiative as we will be able to bi-directionally combine data and prediction flows from the models developed with the created **ToxBank** data and analysis tools and those created on all projects. **ToxBank** will also as a result be able to support strong knowledge flows and integration between both experimental and modelling groups.

The incorporation of systemic toxicology ontologies in **ToxBank** will provide semantic capabilities to link concepts, experimental results and relationships between chemical and biological entities. Once in place, advanced inferencing tools may be used to make complex queries and pose new questions across data and models, which would not be possible with a traditional data description approach alone, and increases the chance of new insights and

discoveries. Incorporation of ontologies also reduces the costs involved with data management resources as the number of data sources are increased thus helping to achieve sustainability goals and industry adoption (*Anonymous, 2009*). **ToxBank**'s ontologies will be based on public standards taking into account collaboration with new ontology developments such as the OBO Foundry so that subsequent extensions, aligned with other biological, chemical and biomedical ontologies are supported in an ongoing manner, including after the project.

Alternative *in vitro* assays need to be well validated, with clear links to known toxicity in humans and animal models. Since molecular mechanisms of toxicity are not always understood, the links between *in vitro* assays and *in vivo* toxicity can only be established by a clear 'audit trail' based on compounds known to cause toxicity. This is the function that will be served by our Gold Compound collection as documented in detail in the TBGCD. This database will provide a quality data source on the physical collection of compounds with known, well-characterised toxicity, which can be distributed to sister projects under this and related EC programs for development of *in vitro* assays. Furthermore selection and analysis tools will be provided for compound subset selection from this 'gold standards' set for particular research project goals, thus strengthening the experimental design of R&D programs, including new programs.

Successful development of predictive toxicity assays requires knowledge of an array of disciplines from chemistry and the purification of reagents, to cell biology and the characterization of cell lines, to pharmacology and the assessment of ADME properties, to statistics and the analysis of patient data, and more. It is virtually impossible for any one person or laboratory to possess this breadth of expertise. The subjective impact of the TBGCD, therefore, is to ensure that the compounds and data selected as gold standard have been vetted by the appropriate experts to ensure that the information meets stringent standards of quality. Hence, this outcome of the project will have a beneficial impact for its pivotal role in harmonization of the data on high-quality compounds and their selection in predictive toxicology, and use of the related data for more reliable toxicity assessment. Involved stakeholders are regulators, industry, and scientists.

The establishment of a centralised physical compound repository will provide a standardised set of 'gold standards' test chemicals for uniform use across the research projects. The analytical facilities will fully characterise test chemical physico-chemical properties, isomerism, purity and stability thus ensuring the use of quality chemical materials in program assays. Such repository operations will increase the likelihood that methods will be developed on the program that are sustainable and can be advanced to validation. Furthermore, the careful analysis of the test chemicals and their data would hopefully prompt other European projects to reach similar quality standards, with a beneficial broader impact on other research studies dealing with toxicity of chemicals.

From a strategic point of view the repository will open new avenues because it will represent a resource which can be extended for other future predictive toxicology projects. Furthermore,

ToxBank will establish links with the parallel repository of information and chemicals within the US EPA ToxCast and Tox21 initiatives. Thus, the impact of this strategy both strengthens pan-European research activities and transatlantic collaboration.

The impact of the **ToxBank** Cell and Tissue Bank information resource will be to provide a quality source of Biomaterials required by the developers of *in vitro* assays and bioreactors on the program projects. This will be valuable for academic and industry researchers. The incorporation of GCCP and GLP procedures and OECD guidance to develop SOPs in the supply, quality control and use of cell and tissue samples should strengthen the validity of assays and help avoid confounding variables, poor reproducibility or flawed data analysis.

Catalogues of quality-assessed Biomaterials and a Web-based supply chain of qualified Biomaterials suppliers will provide access of research groups to quality-assured supplies of materials. Guidance developed on best practice for materials preparation and handling, traceablity and other quality issues will assure that methods are positioned to meet future regulatory acceptance. In addition to strengthening the systemic toxicity methods studied in the **SEURAT-1** Research Initiative, many procedures will provide a firm framework for extension to other areas of toxicity research, drug discovery and HTS screening.

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Zhao, C, Boriani, E, Chana, A, et al. (2008): A new hybrid QSAR model for predicting bioconcentration factors (BCF).- *Chemosphere*, 73: 1701–1707.

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

COACH

Bruno Cucinelli

4.8.1 Introduction

The **SEURAT-1** Research Initiative is a public-private-partnership based on a unique concept. It is composed of the six collaborative RTD (Research and Technology Development) projects described in the preceding sections, and a coordination and support action, which form a coherent cluster with a common scientific objective. These projects have been selected through a call for proposals of the FP7 HEALTH programme ("FP7-HEALTH-2010-AlternativeTesting") published in July 2009, which defined the objectives for a five year research programme for a set of complementary projects.

As opposed to previous clusters established in European research programmes, a synchronised launch of all projects of the **SEURAT-1** Research Initiative was ensured (same start date and duration, individual project programmes defined as complementary parts of a common global research programme). A dedicated coordination and support action was started at the same time to ensure optimal collaboration between the individual projects, also referred to as the "building blocks" and a consistent approach at the cluster level.

4.8.2 Objectives

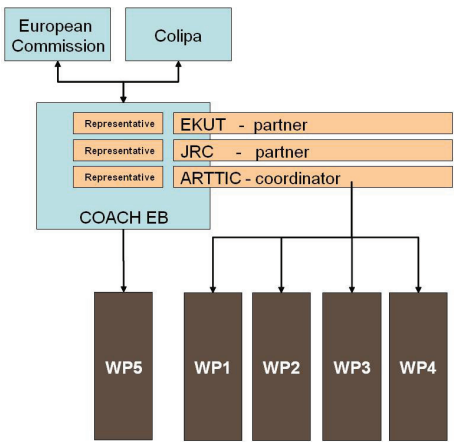
The aim of the coordination and support action **COACH** is to facilitate the collaboration between the research projects at the cluster level through the provision of a Scientific Expert Panel, a cluster level scientific secretariat and a series of support activities and tools described hereafter. **COACH** (Grant Agreement no 267044) started on 1 January 2011 like the six research projects of the cluster, but will have a longer duration of six years to ensure the wrap-up and hand-over of the Research Initiative's outcomes.

The main objectives of **COACH** are to:

- analyze the projects' work plans and progress towards the cluster objectives
- identify opportunities and needs for close collaboration
- organise the cluster annual meetings
- edit the **SEURAT-1** Annual Reports presenting the strategy of the Research Initiative and the progress made
- promote the strategy and results of the Research Initiative to major stakeholders
- disseminate results to the broader scientific community and the general public
- facilitate the operation of the Scientific Expert Panel (SEP) to enable cluster level strategic coordination

4.8.3 Approach

The team leaders of the **COACH** partners form an Executive Board (COACH EB), which decides on the implementation of the **COACH** operations and which reports to the European Commission and to Colipa (*Figure 4.45*). The **COACH** consortium is composed of three partners that work in close collaboration and hold frequent teleconference meetings to monitor the work progress over initial plans, identify possible new requirements and agree on the approach, the responsibilities and the contributions for the actions to be carried out. Each of the three partners is globally responsible for one part of the key activities of the **COACH** workplan that is structured around five distinct work packages as shown hereafter.



WP	Title	Leader
WP1	Facilitation of cluster internal cooperation	JRC / ECVAM
WP2	Preparation and distribution of Annual Books	EKUT
WP3	Organisation of meetings	ARTTIC
WP4	Dissemination	ARTTIC
WP5	Consortium Management	ARTTIC

Figure 4.45 *General structure of COACH.*

The cluster level coordination

COACH provides cluster level coordination and support through the Scientific Experts Panel and the Scientific Secretariat. The Scientific Secretariat is staffed with personnel from the **COACH** partners. The Scientific Expert Panel (SEP) is composed of the six project coordinators (CO) plus seven external experts. These experts have been chosen for their outstanding expertise in the scientific fields covered by this Research Initiative.

Table 4.4 *Members of the* ***SEURAT-1*** *Scientific Expert Panel.*

Participant	Institution	Project
Project Coordinators		
Marc Peschanski	INSERM/UEVE 861, I-STEM/AFM, Evry /France	SCR&TOX
Catherine Verfaillie	Interdepartmental Stem Cell Institute, Katholieke Universiteit Leuven / Belgium	HEMIBIO
Jürgen Hescheler	Institute for Neurophysiology, University Hospital Cologne / Germany	DETECTIVE
Mark Cronin	School of Pharmacy and Chemistry, Liverpool John Moores University / UK	COSMOS
Elmar Heinzle	Biochemical Engineering, Saarland University, Saarbrücken / Germany	NOTOX
Barry Hardy	Douglas Connect, Zeiningen / Switzerland	TOXBANK
External Experts		
Roger Arnold Pedersen	Laboratory for Regenerative Medicine and Cambridge Stem Cell Initiative, University of Cambridge / UK	
Hans Juergen Ahr	Bayer Health Care AG, Wuppertal / Germany	
Emanuela Testai	National Institute for Health, Dept. of Environment and Primary Prevention - Mechanism of Toxicity Unit, Rome / Italy	
Gabrielle Hawksworth	Division of Applied Medicine, University of Aberdeen / UK	
Ian Cotgreave	AstraZeneca Safety Assessment, Södertälje / Sweden	
Catherine Mahony	Colipa (Procter & Gamble), London Innovation Centre / UK	
Derek Knight	European Chemicals Agency, Helsinki / Finland	

The primary role of the Scientific Expert Panel is:

- to identify and propose measures to foster close collaboration between the building blocks of the cluster and to ensure achievement of the cluster objectives towards the long-term goal, which is 'Safety Evaluation Ultimately Replacing Animal Testing' (SEURAT)
- to advise the cluster on scientific matters related to specific topics within the area of repeated dose systemic toxicity testing and to alert (e.g. in contributions to the annual book) on strategic scientific aspects that will have a particular impact on the objectives of the **SEURAT-1** Research Initiative
- to identify knowledge gaps and research priorities and propose solutions
- to elaborate future research agendas

The Scientific Expert Panel makes recommendations on the cluster research programme and on the long-term research strategy (*Figure 4.46*). Decisions, which impact the projects can only be made by the project coordinators, who are in turn bound by their respective Grant Agreements and Consortium Agreements. The Scientific Expert Panel can only make recommendations but not make decisions which could impair or limit a project partner's activities or rights or increases its obligation under the project it participates in. However, this model has been proven in other European research Framework Programmes. Many projects of the FP7 have external independent scientific advisors, and also the European Commission frequently appoints independent scientific experts to periodically review the progress made by the FP7 projects. The role of these independent experts is to make recommendations on the further research orientations of the projects, according to the project's objectives, the achievements obtained, and the international progress in state of the art. The consortia usually acknowledge that these recommendations help them to optimise the outcome of their project and take them into account in their further research work.

The Scientific Expert Panel has two physical meetings per year, which are planned, prepared and followed-up by the **COACH** Scientific Secretariat. In addition, if needed, conference calls are organised on specific urgent focus topics.

The Scientific Expert Panel has also an important role in the preparation of the cluster annual meetings and the writing of the **SEURAT-1** Annual Reports. The typical workflow between the Scientific Secretariat and the Scientific Expert Panel is shown hereafter.

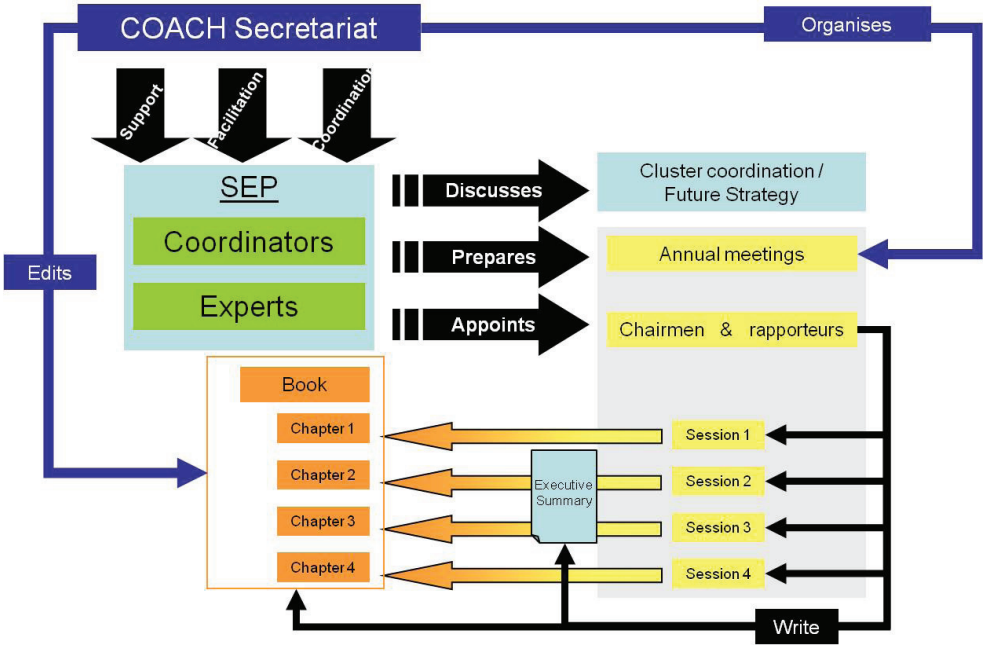


Figure 4.46 *Activities of the Scientific Expert Panel.*

Facilitating exchanges between the participants

The **COACH** Scientific Secretariat organises a number of events and provides electronic tools aiming at facilitating the exchanges between the participants of the cluster, and with external experts engaged in **SEURAT-1** research domains.

1. Annual meetings: Cluster level meetings are organised once a year to give the partners of the cluster the opportunity to meet and to exchange on their research work and on a number of issues that are key for a successful research collaboration.

All organisations involved in the cluster projects are invited to attend, as well as selected experts from industry and research to also foster fruitful exchanges between the cluster partners and eminent experts from outside the cluster.

These annual meetings are composed of keynote speeches and focus workshops and are typically organised over three days always in the same period in the year.

2. Summer schools: The summer schools target in particular the cluster partners' young scientists who carry out the **SEURAT-1** research work. The summer schools have a typical duration of 4-5 days and focus on all scientific areas covered in **SEURAT-1** and the related societal, regulatory and industrial context. The training priorities are defined by a special

task force that is composed of representatives of the cluster projects and facilitated by **COACH**. The summer schools provide an opportunity for young researchers to follow courses given by eminent experts, to present and discuss their own research work and to meet their colleagues from the other research groups. Within the limits of the available capacity, the **SEURAT-1** summer schools will also be opened to young scientists from outside the **SEURAT-1** Research Initiative. The announcements will be made on the **SEURAT-1** public website.

As regards the training of young scientists in the research fields addressed by **SEURAT-1**, **COACH** also provides logistic support for the organisation of hand-on lab training by cluster partners and personal exchange.

3. Ad hoc focus workshops: The **COACH** work programme also foresees the organisation of a series of ad-hoc workshops that will be organised on specific scientific topics according to the upcoming needs of the research initiative. The workshops will normally have a strong focus with the aim to produce specific information required by the initiative. The participants will be selected experts invited for the strong background in the domain addressed by the workshop. The first workshop will focus on mode of action of repeated Dose toxicity.

4. Electronic means: In addition to these events, **COACH** provides and administers a series of electronic tools to support communication, exchanges of information and remote collaboration of the cluster participants:

- A private website: a protected workspace has been set-up on a dedicated web collaboration platform. Each participant has an individual login and can post information on the webspace or read and download information provided by other participants (*Figure 4.47*).
- Mailing lists: A series of dedicated mailing lists is maintained corresponding to the different sub-groups of the cluster
- A conference call service and a web application sharing facility is offered to support the organisation of inter-project virtual meetings

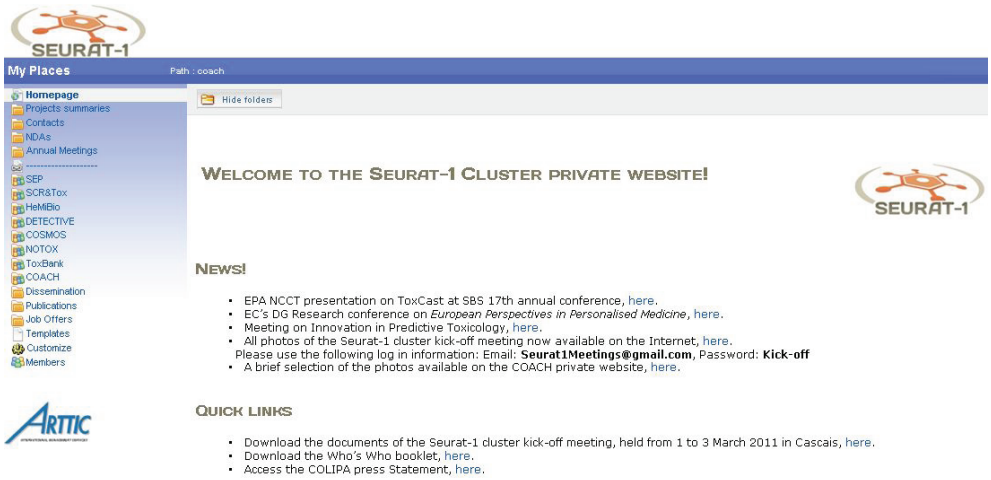


Figure 4.47 *Structure of the private websites organised as a web collaboration platform.*

Information dissemination

COACH has also the aim to promote the research strategy developed by **SEURAT-1** and the results of the cluster's research work. The main target audiences are scientists and stakeholders of this research work and, in particular, regulators, the industry and policy makers, but also the general public. The main dissemination means of the Research Initiative are:

➠ The Annual Report: a series of six annual reports, delivered in the form of books, provide a comprehensive overview about developments in the replacement of animal tests in the field of repeated dose systemic toxicity. The present document is the first volume of the **SEURAT-1** Annual Reports.

➠ The **SEURAT-1** public website: a website informing the public about the Research Initiative, its background and related facts has been set-up at the URL www.seurat-1.eu. It will be regularly updated and offers downloading publications elaborated by this Research Initiative.

➠ Press relations: a first press release has been elaborated in collaboration with Colipa and has been distributed by Colipa. Further press releases and if suitable press conferences will be organised at important milestone dates as decided by the Research Initiative.

➠ Leaflet: The **SEURAT-1** leaflet presents the background, the aims and the research strategy of the Research Initiative. It has been disseminated by mail, will be used on relevant events to inform about **SEURAT-1** and is available for downloading from the public website.

➠ Standard presentation: a PowerPoint presentation on **SEURAT-1** facilitates the presentation of the Research Initiative at scientific and other events and ensures consistency in the communication of cluster partners to the outside world.

4.8.4 Innovation

SEURAT-1 is special in its public-private-partnership approach. The seven projects of the cluster receive funding support from the FP7 HEALTH programme, but are also directly co-funded by the industry through the financial support provided by Colipa, the European Cosmetics Association. The Research Initiative receives a total funding support of 50 M□, from which 25 M□ are provided by the FP7 HEALTH programme and 25 M□ by Colipa. Each consortium has signed a contract ("Grant Agreement") with the European Commission and one with Colipa (the "Colipa Research Agreement"). Colipa's contract is aligned on the FP7 Grant Agreement, which means that the FP7 rules for project expenses, reporting, etc. apply in the same manner for the industry co-funding and hence no notable additional administrative overhead is created.

The **SEURAT-1** Research Initiative has hence built upon an innovative concept as regards the funding model and the structure. Due to its complex work programme and important size, the cluster approach was seen as a better manageable alternative to a big single research project with a 50 M□ grant and a consortium of over 70 organisations.

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4.9 Project and Cluster Activities

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

4.9.1 Project Kick-off Meetings

SCR&Tox: The kick off meeting of *SCR&Tox* has taken place in Evry (France) on 13-14 January 2011. All partners have presented their participation in those work packages that start immediately. The coordination of the work will be greatly facilitated by the presence of a "scientific manager" in the network. Working closely with the coordinator, the scientific manager will be responsible for dealing with all scientific connections between partners, as well as helping with reporting and creating all means for interactions in the network.

Within the framework of *SCR&Tox*, an independent Advisory Board was set up with representatives of the pharmaceutical and cosmetic industry, members of regulatory bodies, as well as an ethical advisor:

- Klaus-Dieter Bremm (Bayer)
- Philippe Detilleux (Sanofi-Aventis)
- Julie Holder (GlaxoSmithKline)
- Gladys Ouédraogo (L'Oréal – Colipa)
- Laura Suter-Dick (Roche)
- Ethics Advisor: Kristina Hug (Lund University, Sweden)

In addition, a large discussion has been devoted to the connection of *SCR&Tox* with the **SEURAT-1** Research Initiative. The activity of *SCR&Tox*, as a provider of biological and technological resources in a first place, and its involvement in toxicity testing only in the second part of the program, was explicitly acknowledged. In that activity, also, the scientific manager will be highly instrumental in liaising with all and making exchanges as fruitful as needed.

HeMiBio: The Kick-Off Meeting of *HeMiBio* was held on 26-27 January 2011, in Leuven, Belgium. Present at the meeting were all partners, as well as three members of the external advisory board:

- Dr. Daniel Duche (Research and Development, L'Oreal Paris), as a representative of Colipa;

➠ Dr. Philip Hewitt (UK and Eurotox Registered Toxicologist; Molecular and Cellular Toxicology (MS-DDT-EMT); Early, Genetic & Molecular Toxicology; Merck Serono R&D from Merck);

➠ Dr. Tomasz Sobanski (European Chemicals Agency – ECHA; Computational Assessment Unit).

The fourth member of the advisory board, Dr. Mehmet Toner (Professor of Surgery (Biomedical Engineering) and Health Sciences & Technology, Harvard Medical School/Massachusetts General Hospital and Director of the BioMEMs Resource Center), was unable to join the meeting.

During the Kick-off meeting, all partners provided a presentation of the technologies available in their labs that pertain to the goals of *HeMiBio*. On day two, studies to be performed in the specific work packages were presented, with a detailed discussion of the goals for year 1, as well as the long-term goals. In addition, breakout sessions were held, with important input from the advisory board members. In breakout session one, the partners involved in the biology/toxicology aspects of the project discussed practical plans for cooperation during year 1 of the project. In a second breakout section, the partners involved in the engineering aspects of *HeMiBio*, discussed the design of the initial and subsequent bioreactors.

As the expertise of the different partners of *HeMiBio* is very diverse, we plan exchange visits between students and postdocs from the different partners to labs of partners with complimentary expertise. These exchange visits will be concentrated during the first 1-1.5 years of the granting period, to ensure a smooth cooperation between partners of *HeMiBio*. In addition, a workshop/summer school will be included during the second year, to provide hands-on intensive experience for students and postdocs from *HeMiBio*, as well as scientists of other **SEURAT-1** projects, or beyond, with technologies used in *HeMiBio*.

At present, considerable activity is already going on with respect to the selection of compounds to be tested in the different development stages of *HeMiBio*. These efforts occur in close collaboration with the other projects of the **SEURAT-1** Research Initiative. A list of selection criteria and a final set of compounds was discussed during a project meeting on 29-30 June 2011.

DETECTIVE: The DETECTIVE kick-off meeting took place at the University of Cologne on 25-26 January 2011. In addition to this first internal consortium meeting, a public event was organised in the evening of the first day. The internal kick-off meeting comprised, after a presentation of the Advisory Board members, presentations of the sub-projects, given by the respective sub-project leaders. After this general introduction with reference to the project and its context, each partner presented his work, organised per sub-project. Parallel working sessions on “Cellular model systems for repeated dose toxicity testing, list of substances,

functional readouts" and on ""-omics" readouts, statistical analysis of biomarkers" were then organised to discuss the practical implementation of the first project phase. The outcome of the working sessions was presented to the plenum resulting in vivid discussions and decided actions. The kick-off meeting concluded with a plenary discussion on the **SEURAT-1** Research Initiative, discussing DETECTIVE as part of it, a feedback on the first Scientific Expert Panel meeting of **SEURAT-1**, and the expected exchanges between DETECTIVE and the other projects of the **SEURAT-1** Research Initiative.

The public session comprised a general project overview presented by Jürgen Hescheler (University of Köln), followed by plenary talks given by Vera Rogiers (Vrije Universiteit Brussel) on "Relevance of repeated dose toxicity testing in risk assessment of cosmetics and their ingredients", by Jos Kleinjans (Universiteit Maastricht) on "Genomics and *in vitro* toxicology", and by Hector Keun (Imperial College of Science, London) on "Prospects for metabolic profiling in the development of *in vitro* toxicity models". The public session was well attended. A project brochure was handed over to all participants.

COSMOS: A kick-off meeting for COSMOS was held on 17-18 January 2011 at the European Commission's Joint Research Centre (EC JRC) in Ispra, Italy. The meeting was attended by all relevant partners and featured a training course on the KNIME Workflow software.

There are already a number of close interactions in the COMOS Project being led at the Work Package level. These include collaborative effort to create the COSMOS database and chemical inventory; joint efforts to develop and update the TTC concept; work to develop grouping and category approaches on a mechanistic basis; considerable effort to develop the toxicokinetic approaches to predict target organ concentration and facilitate *in vitro* to *in vivo* extrapolation.

A key area of exchange of personnel is anticipated to be for the harvesting of toxicological data from the US FDA. In particular it is anticipated that personnel may visit the US FDA from institutions such as the University of Bradford, Liverpool John Moores University and the Institute of Biophysics and Biomedical Engineering, Sofia.

NOTOX: The kick-off meeting of NOTOX was held in Saarbrücken on 25-27 January 2011. All partners participated and after intensive and fruitful discussion, the first steps were planned in more detail. This concerned mainly the work packages dealing with cultivation systems and with '-omics' analyses. In particular, it was decided to start the work with HepRG cells supplied by BioPredic and to start with one selected test compound after negotiations with ToxBank and other projects of the **SEURAT-1** Research Initiative. Spheroid culture of HepRG cells was already successfully initiated in collaboration with InSphero (Zürich) that is now able to supply 96-well plates for the project. Collaboration between the partners "The Netherlands Cancer

Institute" and "German Research Center for Artificial Intelligence" was also started successfully. Partner "Biopredic International" will supply proliferating HepRG cells to consortium members and offers training at their facilities.

NOTOX is actively participating in the workgroup activities guided by ToxBank concerning the selection of test compounds and the planning of data handling within the consortium.

ToxBank: The ToxBank project was kicked off through a combination of virtual meetings held in January and February 2011 and face-to-face meetings held in Cascais, Portugal on 28 February and the 3-4 March 2011. The main focus of these meetings was on activities to be carried out during the first 12 months of the project.

During these meetings, basic issues such as selection of tools for communication, project support and collaboration were discussed. Mailing lists for the ToxBank consortium as well as for the two cross-project working groups (Data Analysis Working Group and Gold Compounds Working Group) were introduced. Particular attention was paid to portal solutions that would allow unified access to a large suite of tools and that could integrate the ToxBank website with project support and communication tools. An ideal solution would provide support for various communities and groups (e.g. the ToxBank consortium and internal working groups, but also the above-mentioned cross-project working groups) and role-based content delivery.

Among the most extensively discussed topics was the task to gather user requirements. For all infrastructure established by ToxBank, the user requirements need to be clear before designing the tools. The ToxBank consortium decided to follow a contextual design methodology involving site visits to partners in the **SEURAT-1** Research Initiative and face-to-face interviews with programme scientists, discussing what they are doing and why they are doing it.

Another important and heavily discussed topic was compound selection criteria. The current status, established in the respective cross-project working group as well as feedback received from the **SEURAT-1** consortia, was reviewed, key issues were analysed, and subsequent steps to be taken were discussed.

Since the design of the ToxBank infrastructure depends on the outcome of the requirements analysis that is in progress during 2011, short-term solutions for data management and provision of information on Gold Compounds were agreed upon during the cluster kick-off meeting in Cascais (see below). For the facilitation of compound selection, a combination of Google Spreadsheets and a Semantic Media Wiki was agreed on. Use cases and user interfaces for a) data uploading, b) protocol uploading and c) searching for information were subsequently designed at a 3 day meeting held at the IRFMN in Milan in June.

4.9.2 Cluster Kick-off Meeting of the SEURAT-1 Research Initiative

The cluster kick-off meeting was held on 1-3 March 2011 in Cascais, Portugal. The meeting was opened by J. Büsing (DG RTD) and R. Taalman (Colipa), representing the two co-sponsors of the **SEURAT-1** Research Initiative, namely, the European Commission, through its 7th Framework Programme (FP7), and Colipa, the European Cosmetics Association. They warmly welcomed the roughly 100 scientists from more than 70 European research organisations that participated in the meeting, and that will collaborate over the next 5 to 6 years. The **SEURAT-1** Research Initiative will focus on developing methods for assessing repeated dose systemic toxicity without using animals, ultimately supporting better safety assessment. It comprises six complementary research projects (*Scr&Tox*, *HeMiBio*, DETECTIVE, COSMOS, NOTOX, ToxBank), supported by a Coordination Action, COACH. This is the first time that such a cluster model has been used within the FP7 programme, and the co-financing by Colipa also represents a novel type of public-private partnership initiative.

Both Büsing and Taalman expressed that they were very much looking forward to a close collaboration between the 6 research projects, and COACH, to ensure that the ambitious goals of the cluster could be achieved in time and with maximum impact. They also recognised that the expectations of the stakeholders in this Research Initiative were high. However, the development of non-animal repeated dose toxicity testing solutions represents a major scientific challenge and thus the launch of the **SEURAT-1** Research Initiative can only be seen as a first step in a longer-term research and development programme entitled, Safety Evaluation Ultimately Replacing Animal Testing (SEURAT).

R. Taalman further introduced the **SEURAT-1** Research Initiative "Towards Replacement of Repeated Dose Toxicity Testing", and W. Dekant (University of Würzburg) presented his ideas on "Use of *in vitro* toxicity data in risk assessment of chemicals: Facing the challenges". Thereafter, each of the projects was presented by the respective coordinators. B. Cucinelli (Arttic) described how COACH was going to facilitate cluster synergy, while V. Rogiers (Free University of Brussels) gave her views on what could be the expected outcome after 5 years. A view from the pharmaceutical sector was provided by I. Cotgreave (AstraZeneca), who looked at key issues that needed to be addressed in working on a longer term strategy (optimal coordination through the identification of common goals, agreements on common interfaces between the projects, development of exchange programmes). The day's proceedings were concluded with a presentation from H. Spielmann, who described the AXLR8 project, and how the EU research programme might transition towards a more pathway based approach to toxicology.

After setting the stage on the first day, the second day focused on reviewing the state of science to give context to the **SEURAT-1** Research Initiative. The speakers identified gaps in current knowledge and recognised where the scientific contributions from the cluster would be

able to set the foundation for a paradigm shift in the risk assessment of chemicals. M. Schwarz (University of Tübingen) opened the morning session by describing selected examples of toxicological modes-of-actions and related biological events/mechanisms specifically related to repeated dose systemic toxicity. H-J. Ahr gave his own perspective on the challenges that will have to be faced in attempting to predict systemic toxicity without resorting to animal testing. Understanding and describing toxicological modes-of-action was recognised as a valid research direction to take, but much of the required knowledge is still lacking. Reducing complex biology in this manner to realise practical test systems that don't miss key processes and effects will be difficult. C. Mahony (Procter & Gamble) described how safety assessment of chemical ingredients in cosmetics is carried out today, and how the current practice might change if pathway based alternative approaches became available. Virtual liver approaches, based around computational models that capture key physiological/toxicological processes in a tissue segment, were outlined by J. Hengstler (Leibniz Research Centre), where, for example, tissue regeneration could be emulated and shown to explain some differences between *in vivo* and *in vitro* results. This is a particularly relevant topic for the Research Initiative since hepatotoxicity is a focal point for many project work packages.

The afternoon session commenced with a talk by M. Cronin (Liverpool John Moores University), who outlined the possibilities of integrating *in vitro* findings with mechanistic information from chemoinformatics and computational modelling, and showed how both chemical structural features and biological activity data could be used to form chemical categories associated with specific modes-of-action. B. Hardy (Douglas Connect) tackled the issue of the definition of an ontology for toxicology, based around mode-of-action concepts, and summarised the outcome of a workshop held in late 2010 on this theme. G. Apic (CCN Ltd.) showed a sophisticated bioinformatics platform that her company had developed to link chemical structure with both pathological outcomes and associated mechanisms. R. Pedersen (University of Cambridge) provided insights into how genetic engineering of stem cells could provide a powerful tool for detecting and investigating toxicity *in vitro*, while G. Stacey (*NIBSC*) discussed the challenges in developing stem cell culturing and differentiation protocols to achieve standardisation and reliability. The day's proceedings concluded with a presentation by A. Kopp-Schneider (DKFZ), who outlined clear recommendations for how to design effective *in vitro* concentration-response experiments, and how to correctly analyse data and report results.

During the discussions, a number of questions were raised that were considered important to address, as follows:

- Currently *in vitro* test systems and *in silico* methods fail to capture complex systemic processes seen *in vivo* such as multiple cell-cell interactions. How can this complexity be addressed and how far can 3D tissues and systems biology modelling go to bridge the gaps?
- How can *in vitro* results be converted into *in vivo* predictions taking into

account differences in biokinetics, and how can metabolism be faithfully incorporated into *in vitro* systems?

➠ How can the time and frequency of chemical treatment/exposure *in vitro* be selected to reflect the *in vivo* situation?

➠ How can genetic, epigenetic and life-stage variations between humans be addressed by *in vitro* systems, so that sub-population susceptibility can be addressed effectively?

In parallel to the plenary talks during the second day, two small workshops took place to deal with cross-cutting topics of i) common data management and analysis and ii) selection of reference ('gold') chemicals. The project ToxBank has established two working groups, dealing with these topics, and these open workshops gave the possibility to inform partners of the objectives of the working groups, how the various issues were going to be tackled, and how the projects/partners might contribute to the work, so that most needs and expectations could be met. A particularly hot topic that needed talking through was the establishment of specific criteria that could be used as a basis for chemical selection. Since the needs and research focus can vary significantly from one research group to another across the **SEURAT-1** Research Initiative, many contributors had different views on what criteria should be taken into account, and how they should be weighted. However, most agreed that one very important criterion for test chemical selection was the availability of *in vivo* data, both in animal and human if possible, and, in addition, well-documented evidence related to the mode-of-action. The ToxBank working group should take on board all the suggestions put forward and work towards defining a selection process and preliminary set of chemicals before the summer.

The third and last day of the kick-off meeting was entitled 'Looking Ahead', and aimed at summarising the discussions of the two workshops held on the previous day, and to get the views of an expert panel on issues that had been raised during the meeting, and to hear their views on how to move forward together. Some key points discussed were as follows:

➠ The need for a better way to do safety assessment without the use of animals which doesn't only identify toxicological hazard, but which can also characterise it in a quantitative manner, thereby delivering activity thresholds (e.g. DNEL) or points-of-departure that are needed for risk management.

➠ The exposure aspect and the need for biokinetic prediction tools to extrapolate from the *in vitro* situation to the *in vivo* scenario – this is an essential component of any new human safety assessment paradigm.

➠ To drive scientific progress, we need to shift the emphasis away from organ specific apical effects and concentrate more on common up/mid-stream events and toxicity pathways at the cellular and molecular levels.

- In conceiving and designing *in vitro* test systems, a fit-for-purpose approach needs to be followed, whereby the aim is to capture specific toxicity pathways and modes-of-action within a system.
- The establishment of well defined and documented standard operating procedures early on in the life of the cluster to capture and transfer knowledge efficiently, in particular in the area of stem cell culturing and differentiation.
- Best practice needs to be shared between labs regarding the design and execution of concentration-response experiments to avoid mistakes of the past.
- Compliance with data reporting standards that will be proposed in the cluster will be critically important for inter-comparison and meta-analysis of results.

There was a wide agreement between panel members and participants that an overall objective of the cluster should be based around a proof-of-concept, namely, to show how a pathway/mode-of-action based approach can deliver methods for predictive toxicity that can be used for safety assessment purposes. It was proposed that the Scientific Expert Panel of the Research Initiative would be best placed to elaborate on these ideas and make some practical proposals for action.

The kick-off meeting was thereby concluded. The next annual meeting for all the 7 projects of the **SEURAT-1** Research Initiative is planned for February 2012.

4.9.3 Proof-of-Concept Approach

The proof-of-concept idea was discussed further in the meeting of the Scientific Expert Panel that followed the cluster kick-off meeting. It was agreed that such an exercise would be very valuable to promote cross-cluster interaction and would be a very useful means of communication and outreach to engage stakeholders and potential end-users. The aim should be to demonstrate proof-of-concept at three different levels, namely, (i) identify and describe a mode-of-action, (ii) predict toxicity, and (iii) support a safety assessment decision.

The first level will require the coordination of scientific expert input to define and document chosen toxicological modes-of-action associated with repeated dose target organ toxicity, most likely the liver. This could be supported, for example, through a dedicated workshop organised by COACH. Proof-of-concept at the second level should be centred around a feasibility study to demonstrate an integrated approach to predict selected types of repeated dose target organ toxicity, based on the assembly of complimentary tools and test systems developed within the cluster, and following the mode-of-action approach elaborated in the first level. To tackle the third level, the Scientific Expert Panel, together with relevant cluster partners, will need to formulate a number of safety assessment scenarios where data/information on a substance,

generated applying methods used in the **SEURAT-1** Research Initiative, could be used to support decision making. These scenarios might be further illustrated with selected case-studies, if appropriate and feasible. The ultimate aim should be to demonstrate prototype assessment frameworks, based on a mode-of-action approach, that can exploit the knowledge and tools derived from the cluster.

4.9.4 Expected Progress within the First Year

The first year of activity of *SCR&Tox* is entirely dedicated to the provision of biological and technological resources, and is integrated in activities that will altogether span over the entire first half of the network.

During the first year, *HeMiBio* will develop methods for isolation of the different cellular components using cell surface antigens from mixed iPSC progeny and test the ability to apply UpCyte® technology to expand the cellular components derived from iPSC or from primary livers, without dedifferentiation. In addition, we should be able to prove that introduction of promoter or toxicity assessment cassettes using ZFN-HR is possible by the end of year 1 of the proposal. Such cells will then be provided to the engineering partners to start testing the creation of the different bioreactor designs. The first bioreactor to be developed is the 2D micropatterned bioreactor, and the initial packed-bed bioreactor. These should be in working order by the end of year 1, and available for evaluation of the effect on cell phenotype and initial toxicity screenings during year 2.

Aside from generating the biological components for the bioreactor, *HeMiBio* will during year 1 evaluate the function of commercial sensors for pH, O_2 and glucose, as well as start developing appropriate microsensors for real time assessment of the health of hepatocytes, such as for instance sensors for alanine transaminase (ALT) or LDH. We will also work out a solution for integration of these sensors in future bioreactors. The possible designs for the final bioreactor will also be completed, including initial assessment of the materials to be used in the final design.

The DETECTIVE work plan is divided into different phases according to the availability of test substances and cell systems and to the readout systems used. These will have impact on the type of data analysis to be carried out. The consortium has begun work with the cell systems currently in use within the consortium (heart, liver and kidney). Quality control of the applicability of these cell systems will be carried out in the first months, using both functional and “-omics” readouts, which will provide more insights into the physiological quality of the cell systems used, as well as their suitability to detect repeated dose toxicological modification. Protocols for repeated dose toxicity testing, for sample preparation including shipping to the partners performing “-omics readouts” and for processing of proteomic experiments will be prepared in the first 6 project months. Similarly, cell lines and protocols are transferred to the

partners performing the functional readouts. First dose response curves are then planned to be available for both functional and "-omics" readouts by month 9. The related analysis of these first functional and "-omics" datasets will be carried out until the end of the first project year. The design of the internal database, interoperable with the **SEURAT-1** Research Initiative database, is also planned by the end of the year.

The COSMOS project will create a database for the toxicological information, which will be populated with initial data within the first year. It is intended that this will be in a form that can be accessed by ToxBank. Furthermore, initial harvesting of toxicological data will have taken place and a chemical inventory will have been created and will be partially populated with high quality chemical structures. The ILSI-EU Expert Groups will be created to assess the needs of TTC for cosmetics and initial groupings of chemicals for endpoints relevant to repeated dose toxicity will have been established. Additionally, the requirements for PBPK and kinetics modelling will have been laid down. Finally, initial workflow technology will have been created with the possibility for a simple illustrative KNIME workflow for chemical grouping.

In the first year, NOTOX will establish cultivation of HepRG cells in various reactors. First, epigenetic profiles will be created. Proteomic analysis will start with the extracellular proteome and identification of target proteins for quantitative analysis of e.g. signalling cascades using samples created by project members. Created 3D organotypic cultures will be investigated by optical and electron microscopic methods available. Data treatment methods to improve image quality and to extract characteristic features will be set up. Bioinformatic and mathematical large-scale modelling methods to model biological systems to be used in NOTOX, will be established.

At the beginning, ToxBank will investigate the user requirements for the Data Warehouse and will have the system design, architecture, Application Programming Interfaces (APIs), and the operating procedures for the up- and downloading of data available at the end of the first year. The selection criteria and SOP's for data quality control, acceptance, processing and analyses will be defined with respect to the Gold Compounds, to be studied in the projects of the **SEURAT-1** Research Initiative. Furthermore, the requirements for the repository, as well as the distribution of test chemicals and the various biological samples, will be clarified in the initial phase of the project.

As for all research programmes, the launch phase of **SEURAT-1** was a critical step for the set-up of the required infrastructure, the start of activities and the creation of the required group dynamics. These are tasks of COACH, and a number of important results have already been achieved:

> ➠ The Scientific Expert Panel has been constituted and three SEP meetings have been organised. One of the key results was the definition of a long-term research strategy (see chapter 3), initially drafted by the COACH team that was discussed, refined and adopted by the SEP.

➠ A cluster kick-off meeting was organised from 1 to 3 March 2011 in Cascais, Portugal. About 100 participants from the cluster projects and some selected external experts came together for three days of fruitful exchanges. A first version of a “Who is Who” booklet was prepared and disseminated during this meeting.

➠ COACH partners supported the focus task forces set-up on test compounds and data management and currently contribute to the establishment of the required agreements for exchange of data and information at the cluster level.

➠ A visual identity (logo, document layout, website design, Annual Report layout design) was created and the dissemination material mentioned in the previous section was prepared.

➠ A homogenous training programme at the cluster level was established based on the training activities planned by the individual projects.

➠ Electronic means facilitating the communication, exchange of information and remote collaboration, and in particular the **SEURAT-1** private web-space, have been set-up.

➠ COACH partners have also ensured liaison with other research initiatives, such as AXLR8 and other research projects in the field of human safety testing.

The next important steps will include the preparation of the second annual meeting, the preparation of the Annual Report 2012 and the implementation of the **SEURAT-1** training programme.

4.10 Cross-Cluster Cooperation

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

4.10.1 Introduction

Cross-cluster cooperation is being supported by the development of a cluster-level agreement on the sharing and management of proprietary and confidential data, protocols, chemical and biological information between cluster partners, so as to optimise opportunities for collaboration and to accelerate knowledge flows for innovation breakthroughs by the cluster in integrated data analysis, biomarker discovery and test system development. Two cross-cluster working groups were established at the start of the **SEURAT-1** Research Initiative in the areas of data analysis and compound selection; it is expected that this approach will be extended to cover other common interest areas such as biomaterials and stem cell differentiation.

4.10.2 Working Groups

Data Analysis Working Group

ToxBank established a cross-project Data Analysis Working Group (DAWG) open to participation by representatives from all **SEURAT-1** projects. The DAWG group discusses on an ongoing basis best practices, standards and common approaches for programme data management and analysis including topics such as vocabularies, protocols, ontologies, statistical analyses, and integrated data analyses. The group will also develop ideas and new approaches for data analysis required by emerging research activities carried out in the Research Initiative, e.g., the extraction of biomarkers from “-omics” data. We expect that the DAWG will a) encourage transfer of data-related expertise and tools between projects, b) support cross-project discussions on optimizing data uploading and formats, c) help form agreement on standardization where possible, d) discuss and plan the challenging issue of different levels of modelling from local to fully integrated analysis across all data obtained in the **SEURAT-1** Research Initiative, and help design out inefficiencies and optimise experimental design, e) help avoid duplication and optimise alignment on data analysis. Regular virtual discussions are held between the DAWG group members. Discussions with experimentalists developing assays will also be facilitated to agree on formats for documenting experimental protocols including exposure, cell type, dose-response representations, and time intervals, which will be linked and uploaded along with datasets.

Gold Compounds Working Group

A cross-project Gold Compounds Working Group (GCWG) was established by ToxBank at the start of the Research Initiative to collaborate on compound selection. The goal for the group is to achieve consensus across the stakeholders in the **SEURAT-1** Research Initiative on the selection criteria for accepting test compounds and the selection of prioritised compounds for testing on project assays. In order to support early development and testing work carried out in the **SEURAT-1** projects, a preliminary short list of candidate test compounds was established during spring 2011 for different endpoints including hepato-, renal- and cardiotoxicities; a criterion for these compounds is a preference for previously well-studied compounds for which a good understanding of mechanisms of action for phenomena, such as cholestasis and steatosis, exist.

After the assembly of this initial list of candidate compounds and their grouping according to categories, the working group will ask for final comments from the other participants in projects of the **SEURAT-1** Research Initiative and the SEP before final acceptance of compounds into the ToxBank Gold Compound Database (TBGCD). In addition, the compounds in the database will be subject to ongoing review in order to detect and flag inconsistencies in the biological data. A subsequent development will be the incorporation of a mode-of-action framework to the test compound set, taking advantage of the workshop on mechanistic aspects being organised by COACH (see chapter 4.11.2).

Proposals for other Working Groups

A discussion about potential additional working groups focusing the broad expertise from specific areas available within the cluster was initiated during the cluster kick-off meeting in March 2011 in Cascais, Portugal. Stem cell differentiation, the identification of biomarkers and compounds, and the development of *in silico* techniques were identified as areas for setting up additional Working Groups. COACH will support the establishment of new Working Groups as soon as a number of researchers from the **SEURAT-1** cluster projects express their interest to participate.

4.10.3 **Materials and Computational Tools**

Biological Materials

Building on their stem cell-based research and development, *SCR&Tox* will be particularly able to support other projects with biological resources of human cells of different genotype, phenotype and stages of differentiation, as well as with technological resources for scalable cell production and high throughput screening and methodologies applicable using those resources for cell analysis and cell function assessment *(Figure 4.48)*. Dissemination and

technological transfer will be accessible to members of all projects of the **SEURAT-1** Research Initiative. Conversely, demonstration of the value of those resources and methodologies for long-term toxicity testing on the *SCR&Tox* industrial-scale platforms will be based upon the identification of specific toxicity pathways of interest by other projects such as DETECTIVE and NOTOX. *SCR&Tox* will rely on the characterization by other projects of those signalling systems, relevant biomarkers and discrete endpoints, as well as the test compounds selected and prepared in the common ToxBank data warehouse, in order to implement its large-scale, long-term, multi-parametric testing.

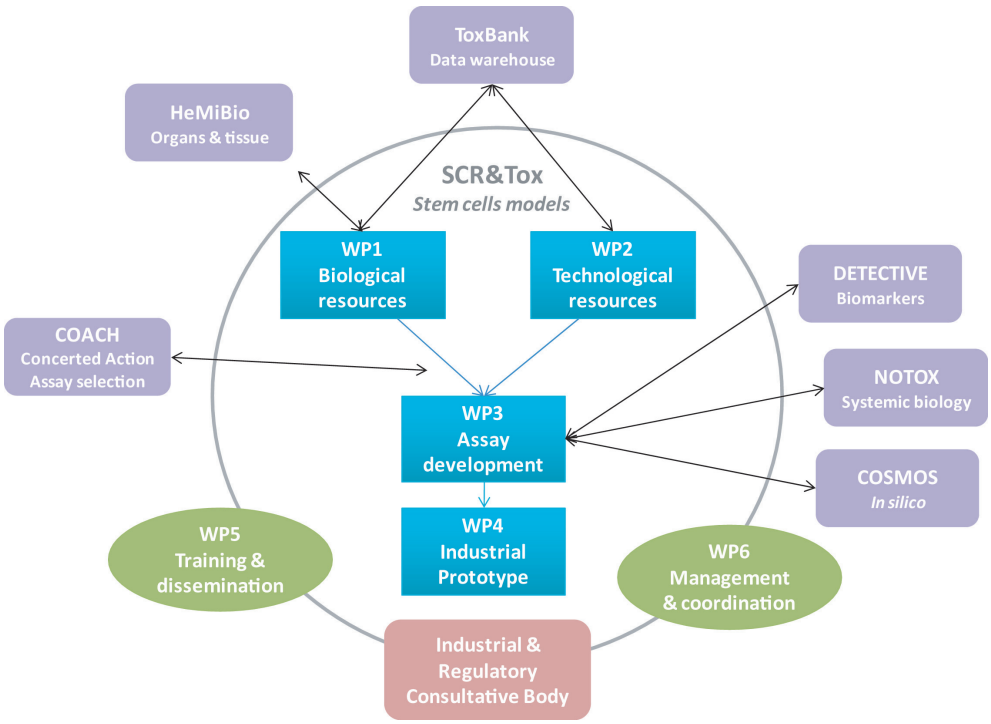


Figure 4.48 *Cooperation between SCR&Tox work packages and other cluster projects.*

Biomarkers

Within the context of the **SEURAT-1** Research Initiative, the DETECTIVE project will establish a biomarker development pipeline for identifying markers of repeated dose toxicity in human *in vitro* models. DETECTIVE's impact will be significantly increased through successful interaction with the other building blocks. The mutual interdependencies are illustrated in *Figure 4.49*.

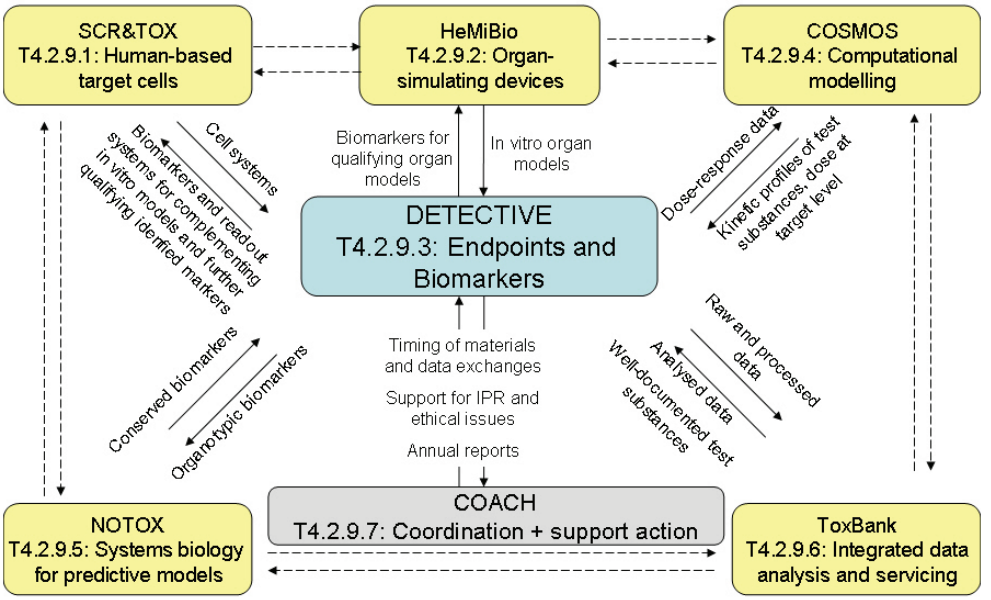


Figure 4.49 *Interactions of building blocks of the* ***SEURAT-1*** *Research Initiative supporting biomarker discovery.*

DETECTIVE will both integrate the output of the other building blocks into its own work, as well as provide other building blocks with the results of its research. From *SCR&Tox*, the DETECTIVE project will incorporate optimised model systems as they become available and after having assessed their suitability for the planned readouts. Prior to availability of new model systems, existing systems will be used by the project. In return, DETECTIVE will provide verified organo-specific biomarker information to support assessment of cellular model systems under development. Analogous interactions are foreseen with *HeMiBio* that will implement and adapt multi-organ cellular devices for long-term toxicity testing.

COSMOS will provide information about thresholds of concern and predictions of target organ concentrations and accumulative effects that are needed for DETECTIVE to test relevant concentrations of the test substances. In return, dose-response data established in DETECTIVE will be fed back in order to support modelling activities. DETECTIVE will exchange information with NOTOX regarding human, organotypic biomarkers for comparative analysis with biomarkers conserved across species.

ToxBank plays a key role in conducting tests in all building blocks through identifying suitable test substances with known modes of action and available clinical data and functioning as a central repository for the test chemicals. It will also function as a data warehouse and analysis centre for raw and processed data from DETECTIVE and NOTOX (subsequent to the

agreement on Intellectual Property Rights cover by the cluster-level agreement), which could support integration of different evidences into cluster-level biomarker discovery. Analysed data from ToxBank will be fed back into the DETECTIVE and NOTOX projects to adapt experiments accordingly.

Systems Approaches

As they become available, cells will be applied preferentially derived from hESC cell lines established by *SCR&Tox* and *HeMiBio* and cultured in devices developed in *HeMiBio* will be applied. Test compounds will be selected in joint agreement with all other projects coordinated by ToxBank. Data of varying depth will be collected at appropriate intervals to create a most comprehensive coverage of toxicologically relevant data on activity and related structure. Large-scale cellular networks developed by NOTOX will be integrated in a spatio-temporal liver organ model for PBPK simulation studies in collaboration with COSMOS. Data will be tailored for mutual use and adapted to data handling in the ToxBank data warehouse. NOTOX partner CCN (“Cambridge Cell Networks Ltd”) will be strongly involved in the link to ToxBank to collaborate on the application of ontologies to ToxBank services providing biological effect and pathway views on the data.

Modelling

COSMOS is a computational project producing databases and models. It is anticipated that its strongest technical cooperation will be with ToxBank to support the provision of access to databases and models to the cluster.

It is anticipated that COSMOS will lead the creation of a chemical inventory for cosmetics ingredients. ToxBank will enjoy integrated access to this inventory to assist in, for instance, selection of compounds for testing to maximise the coverage of chemical space. Further, COSMOS will provide access to the publicly available COSMOS toxicological database, although access to confidential data will need to be restricted. To enable effective and interoperable resource interaction, ToxBank and COSMOS are collaborating on the necessary computing interfaces and standards for communications between the developed systems. The interfaces will for example seamlessly allow for a ToxBank user to search the COSMOS database. This will support the integrated data warehousing and analysis functionality of ToxBank.

There are numerous other opportunities to link the modelling efforts in COSMOS with other projects in the **SEURAT-1** Research Initiative. Most important will be the opportunity for a cluster level exchange of knowledge regarding mechanisms of toxic action. The COSMOS project will benefit from information on mode of action to underpin the grouping efforts for

read-across. In addition this information will be invaluable for the definition of molecular initiating events associated with the various adverse outcome pathways. Likewise, data can be provided to the **SEURAT-1** Research Initiative in an attempt to test and break models.

4.10.4 Development of Risk Assessment Tool

Cluster-level collaboration will be required for the development of tests and tools that can be applied to future risk assessment. *Figure 4.50* shows the anticipated collaboration between the cluster projects required for the derivation of a NOAEL for liver toxicity, based on an integrated strategy for the *in vitro* evaluation of repeat-dose liver toxicity. It combines stem cell-derived cellular systems with modern technologies in biomolecular toxicology and computer modelling.

In the first instance, it is considered crucial to identify the major molecular pathways and measurable parameters involved in systemic liver toxicity, together with a set of compounds that show clear hepatotoxic effects. To this end, three closely linked projects tackle the critical steps of compound selection and parameter analysis. ToxBank is concerned with compound selection for toxicity endpoints, and integrated -omics and functional analysis. ToxBank analysis facilities will closely interact with the identification of molecular pathways relevant for human long-term systemic toxicity (NOTOX), and the identification of suitable -omics and functional biomarkers for toxicity (DETECTIVE).

Two separate projects will tackle the development of suitable cellular and/or organ models in which measurement of the defined liver-specific toxicity parameters may give a good indication of the expected human liver toxicity *in vivo*. *SCR&Tox* aims at delivering human hepatocytes through stem cell technology. Functional cells that could be derived from stem cells will be the major cell type for loading into a liver-simulating device that will be developed under the *HeMiBio* project. Once the liver-simulating device is sufficiently standardised, it will be repeatedly exposed to different (low, medium, high) concentrations of the chosen hepatotoxic compounds. Dose-response curves of the compound-induced changes of the identified biomarkers and/or molecular pathways relevant for long-term liver toxicity will be determined in order to establish the *in vitro* “human No Observable Adverse Effect Concentration” (hNOAEC).

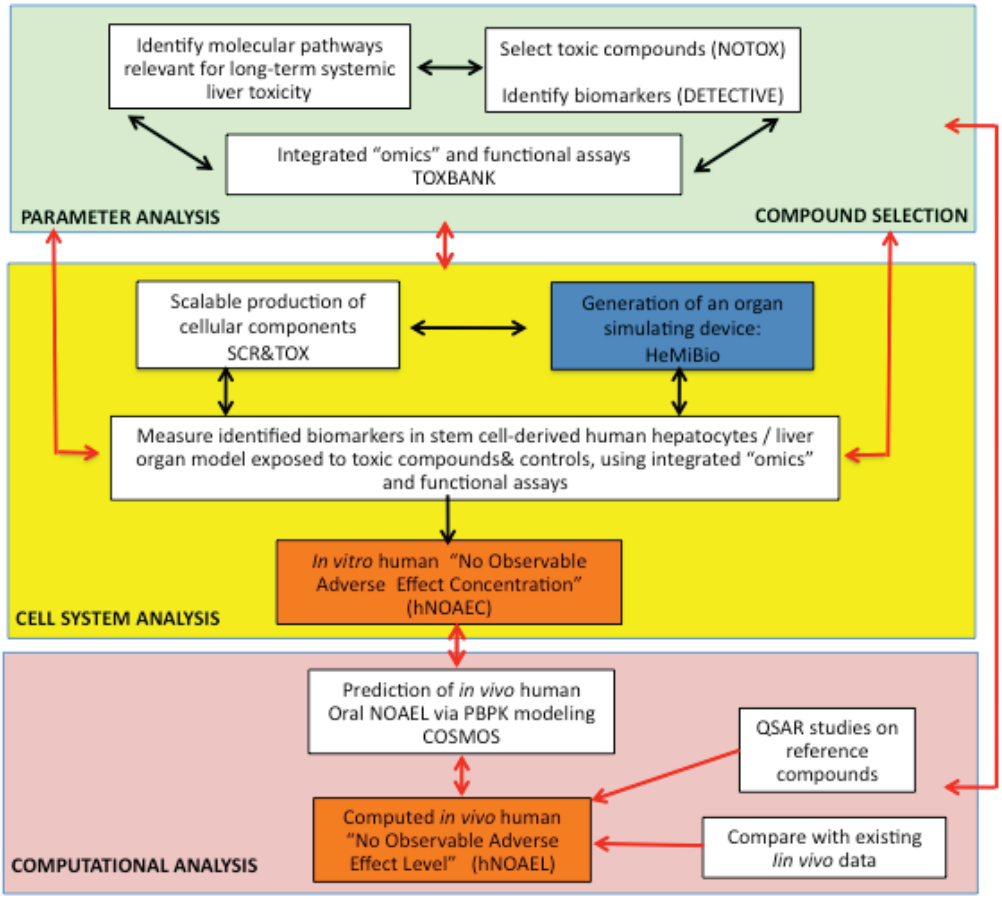


Figure 4.50 *Derivation of a NOAEL for liver toxicity.*

In a third module of the strategy, computational techniques will be applied to estimate the *in vivo* "human oral NOAEL (hNOAEL)" value. This can be done by applying Physiologically Based Pharmacokinetic (PBPK) computational modelling to the hNOAEC value measured in the above cell/organ system(s) and by taking into account an appropriate uncertainty factor for the *in vitro-in vivo* extrapolation (COSMOS). Simultaneously, Quantitative Structure-Activity Relationship (QSAR) studies should be performed on the predefined set of hepatotoxic compounds (COSMOS, ToxBank). The computed $hNOAEL_c$ can then be compared with already existing *in vivo* data and in case satisfactory results are obtained, the $hNOAEL_c$ could be envisaged to be used in risk assessment calculations. As under the *HeMiBio* project the *in vitro* experiments will be carried out on human cells, interspecies variation does not come up for discussion. Assuming that the PBPK modelling already took into account the *in vitro-in*

vivo extrapolation, the ratio of the obtained $hNOAEL_c$ and the systemic exposure dosage (the intraspecies uncertainty factor) needs to be at least 10 in order to consider the compound as being safe with regard to its hepatotoxic effects. Of course additional safety factors can be introduced during the whole process.

Considering the ideal situation that all elements shown in *Figure 4.50* are covered by the 6 projects with respect to liver toxicity, it becomes clear that the *HeMiBio* liver-simulating device supports an integrated approach for a realistic prototyping of an *in vitro* strategy for repeat-dose liver toxicity testing. In the *HeMiBio* project, with liver as the main target organ, attention is given to the "natural" environment of the hepatocytes in the liver, making not only the extracellular matrix an important factor, but also cell-cell contact with endothelial cells, Kupffer cells and stellate cells.

When successful, the concrete prototype proposed in the *HeMiBio* project for liver, can be repeated later onwards for other target organs (*e.g.* heart, kidney, lungs) with the ultimate goal to be able to select the lowest $hNOAEL_c$ and the most sensitive target organ. In this way, the integrated *in vitro* strategy becomes more complex, but also closer to the current *in vivo* approach for repeat-dose toxicity testing.

4.11 Training and Outreach

The COACH Team

4.11.1 Training Activities

SEURAT-1 is organising a training programme aiming at spreading knowledge on the scientific domains covered by this Research Initiative and the related ethical, industrial and regulatory matters. This training programme addresses young scientists, and in particular research fellows from the organisations that are involved in this Research Initiative. When the capacity of the training courses allows it, they will also be opened to external participants.

Training activities are essential for supporting the integration of young scientists and to transfer them knowledge, which is a key for being as efficient as possible in the cluster projects' research and development work. The most important aspects for the young researchers are to be informed about the specific needs and constraints in the application domains of the research results, and to understand the complementary research work that is carried out by the other groups. Besides, these training activities will support strengthening collaboration and creating synergies between research teams from the different projects while demonstrating effective cluster level collaboration.

The **SEURAT-1** training programme is based on the training activities that had initially been foreseen by each of the different cluster projects. However, these training activities have been defined individually during the projects development phase and had consequently different scopes, implementation approaches and timing. COACH therefore prepared a proposal for a common training programme aiming at harmonising the training activities of this Research Initiative. The benefit is obviously to optimise the invested effort and expenses, to increase the potential audience for each course and to offer a richer training programme to the trainees.

The training courses cover the scientific areas that are addressed within the **SEURAT-1** Research Initiative and the related societal, regulatory and industrial context, such as for example:

- *in vitro* toxicity test systems
- modern concepts in toxicology
- industry and regulatory requirements
- ethical aspects
- practical hands-on data analysis

- automation of stem cell culture
- stem cell isolation, characterization and differentiation
- bio-engineering of culture devices

The **SEURAT-1** training task force will specify the training priorities for the coming training sessions. This group will work in close collaboration with COACH for planning the next training events. It involves the training workpackage leaders of each project, namely:

- Sébastien Duprat, INSERM – ISTEM (France), *Scr&Tox*
- Pau Sancho, Hospital Clinic Barcelona (Spain), *HeMiBio*
- Michael Hekke, University Hospital Cologne (Germany), DETECTIVE
- Marjan Vrako, National Institute of Chemistry (Slovenia), COSMOS
- Gordana Apic, Cambridge Cell Networks Ltd, (UK), NOTOX
- Barry Hardy, Douglas Connect (Switzerland), ToxBank

The plan of the future training courses will be presented on the upcoming annual cluster meeting, notified to the **SEURAT-1** partner organisations by email and announced on the website.

The training material produced for the different courses (such as presentations, background material on the different scientific and technological domains) will be collected in a dedicated section of the **SEURAT-1** private website (see below) and made available to all partners, and in particular to “newcomers” who might join the project at a later stage, with the aim of facilitating their integration in the project.

The **SEURAT-1** training programme foresees two types of training: summer schools and hands-on lab training, as described in the following paragraphs.

Summer schools

The main objective of the summer schools is to spread the knowledge from **SEURAT-1** related research domains within and beyond the cluster. In addition, the summer schools provide an opportunity for the young researchers to meet their colleagues from the other research groups, present and discuss their work and also to follow courses given by eminent experts. They are usually composed of lectures by senior scientists and invited speakers, presentations made by young researchers, workshops, and poster sessions.

Four summer schools will be organised in the scope of **SEURAT-1**, from summer 2012 to 2015. The access to the summer school courses will be free of charge for staff from the **SEURAT-1** partner organisations.

The following list summarises the main characteristics of the summer schools:

- Teaching format: lectures, presentations, workshops, poster sessions
- Periodicity: once a year, either in June or in September
- Duration: 5 days (starting at noon the first day and ending at noon the fifth day)
- Expected average audience: about 100 participants

The venue is foreseen at Instituto de Biologia Experimental e Tecnológica - IBET (www.ibet.pt), a large biotechnology research organisation located at Oeiras, Portugal (12 km from Lisbon). IBET has the suitable infrastructure for the courses (classes, labs, conference rooms, conference equipment) and offers to host the **SEURAT-1** summer schools at favourable conditions. IBET are keen to host the **SEURAT-1** training courses, in particular because they are planning to set up a "EU Training Centre for Safety Evaluation", providing a long term offer for training and education in the field of toxicology, safety and preclinical testing.

Hands-on lab training

The main objective of hands-on lab training is to transfer specific skills and expertise to researchers involved in **SEURAT-1**, potential future users and scientists from other research projects. These practical lab training courses will focus in particular on transferring knowledge about state of the art and leading edge technologies and operating procedures to the trainees.

These training opportunities are offered by various laboratories involved in the **SEURAT-1** Research Initiative. The specific objective of these training opportunities, the dates, location, duration, number of trainees depend hence on the offers made, but generally they will address small groups. The participation to these courses will usually be subject to a fee, which will depend on the costs generated for running these courses (e.g. the materials used by the trainee).

The information about these hands-on training offers will be collected by the COACH office, disseminated to the cluster partners and announced on the **SEURAT-1** public website.

The following list summarises the main characteristics of the hands-on lab training:

- Teaching format: hands-on lab courses in partner's laboratories
- Periodicity: no specific periodicity
- Duration: the duration will be variable depending on the training objectives
- Expected average audience: small groups
- Venue: facilities of the partner offering the training course

4.11.2 **Workshops**

Besides the working groups focussing on data management and selection of candidate compounds that will be organised by ToxBank (see chapter 4.10.2) additional workshops will be organised addressing specific aspects of repeated dose systemic toxicity. Participants should be experts in the respective fields, as the intention is to discuss open questions of the field on a high level and provide suggestions for future activities. In principal, the workshops are open for external experts and may be used as a starting point for collaborations between cluster projects of the **SEURAT-1** Research Initiative and other related international activities.

Online Workshop on Open Data: What, Why, How?

This online workshop has been held on 9 May 2011 and was organised by ToxBank. Open Data is one approach to making data more easily re-usable including specifications by the data creator on the terms of the type of re-use. The goal of this interactive virtual meeting was to address misconceptions around Open Data and to answer questions experimental biologists, chemists, and toxicologists had with respect to how Open Data can help their research. Rufus Pollock (Open Knowledge Foundation) and John Wilbanks (Creative Commons) were invited speakers in this workshop. The programme was complemented by a discussion facilitated by Barry Hardy (Douglas Connect) on how Open Data could contribute to the success of EU FP7 projects including OpenTox, ToxBank and **SEURAT-1**.

Workshop on mode of action relevant to repeated dose systemic toxicity

The development of new *in vitro* and *in silico* techniques for the prediction of repeated dose systemic toxicity requires knowledge about the underlying mechanisms. A workshop involving experts from the cluster as well as external experts in the field is planned for 14-15 November 2011 at the European Commissions “Joint Research Centre” (JRC) in Ispra, Italy. The number of participants is restricted to a maximum of 20. The outcome of the workshop should be a report about the mechanisms of repeated dose toxicity. COACH will organise this workshop jointly with the JRC.

Workshop on the use of non-standard test data in the regulatory context of safety assessment of the EU REACH and CLP Regulations

The overall purpose of regulatory efforts in the context of chemical testing is to ensure a high level of protection of human health and the environment. Industry has to ensure that chemical substances are used safely. This is achieved by using information on the properties of substances to assess their hazards both for classification and risk assessment, and hence to develop appropriate risk management measures to protect human health and the environment. If data gaps were identified the registrant should consider the use of non-animal data before any new tests are conducted, such as information from structurally-related

substances (i.e. 'read-across' and 'chemical categories') and predictions from valid (Q)SARs, as new animal studies should only be the last option. However, registrants have to justify these adaptations of the standard information requirements in the registration dossier and provide scientific explanations why the non-standard data is nevertheless adequate, i.e. it must meet the REACH data requirements and be suitable for an adequate risk assessment to ensure the substance can be used safely.

This workshop will focus on best practices in the use of non-standard data for REACH and CLP. It is planned for the beginning of 2012. Experts in risk assessment from industry and academia will be invited. COACH will organise this workshop in conjunction with *SCR&Tox* and with support from ECHA. Additionally, *SCR&Tox* will arrange additional workshops in this field on a yearly basis.

4.11.3 Public Website

The World Wide Web has become a major information channel. The **SEURAT-1** Research Initiative will ensure that an appropriate level of visibility about this initiative is established on the Web and has therefore set-up a public website at the domain address *www.seurat-1.eu*. The aim of the **SEURAT-1** website is to support dissemination of information about this Research Initiative, its strategy and its results, including publications such as the **SEURAT-1** Annual Reports, brochures and leaflets. It 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.

Each of the six research projects that are part of this initiative has its own website to provide more specific details on the project's objectives, scientific work-programme and the consortium partners. These websites can be found at the following domain addresses:

- *SCR&Tox* (Stem Cells for Relevant Efficient Extended and Normalized Toxicology): http://www.scrtox.eu
- *HeMiBio* (Hepatic Microfluidic Bioreactor): http://www.hemibio.eu
- DETECTIVE (Detection of endpoints and biomarkers of repeated dose toxicity using *in vitro* systems): http://www.detect-iv-e.eu
- COSMOS (Integrated *In Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety): http://www.cosmos-tox.eu/
- NOTOX (Predicting long-term toxic effects using computer models based on systems characterization of organotypic cultures): http://www.notox-sb.eu

- ToxBank (Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology): http://toxbank.net

The **SEURAT-1** website has been designed, set-up and is maintained by the COACH coordination and support action. The website design corresponds to a global corporate style created for **SEURAT-1**, which includes the logotype, the colours and fonts that is used for all **SEURAT-1** dissemination material to ensure a consistent professional image. The main sections and features of the website are described hereafter.

Main sections:

Background explains the rationale for launching this Research Initiative, the socio-political, regulatory and scientific context and the urgent needs for carrying out research in the field of human safety assessment solutions.

The Research Initiative provides a comprehensive description of the **SEURAT-1** Research Initiative, including:

- Its vision and strategy
- The underlying scientific concept
- The participant organisations
- A Who is Who database containing short CVs of each of the involved scientists
- Job openings at **SEURAT-1** partner organisations

Cluster Projects contains a short summary description of each of the projects constituting this Research Initiative, contact information and links to the individual project websites that provide more detailed information for each project.

Library contains documents resulting from **SEURAT-1** and links to relevant information related to **SEURAT-1** available from other sources, including:

- "Publications" prepared by the **SEURAT-1** Research Initiative (generally available for downloading), as well as links to scientific publications made by **SEURAT-1** partners
- "Bibliography" provide links to publications that are important references for the **SEURAT-1** Research Initiative (such as brochures, press releases, leaflets)
- "Links" to related projects, initiatives, agencies, organisations
- "Events" contains summary information and links to events organised by this Research Initiative and external events relevant to the research work carried out

in **SEURAT-1** (also indicated in the “events” calendar on the left hand menu)

➠ “News” informs about important announcements of relevance for the **SEURAT-1** research field (the most recent news are also made visible in the News box on the left hand menu)

➠ “Contact” information of the COACH Office.

The website contains some practical features to ease navigation, to find information and to support interactivity for the visitor:

➠ A calendar allowing to quickly browse through the upcoming events of **SEURAT-1** and of any related initiative

➠ A news box showing the three most recent news headlines with links to the complete information

➠ A sitemap providing a complete overview of the website structure

➠ A path indicator showing where the currently visited page is located in the hierarchical structure of the website and allow to return to any of the higher levels of this path

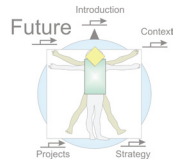
➠ A search tool allowing a full text search in the entire content of the website

➠ An RSS link for visitors to stay posted on the major updates being brought onto the website

➠ A registration form to register in the **SEURAT-1** mail list for printed material, like the Annual Reports

➠ A contact form to get in touch with the COACH office.

The project website will be regularly updated, enriched and evolve along with the cluster activities to reflect the most recent findings and results.



5 PREPARING FOR THE FUTURE

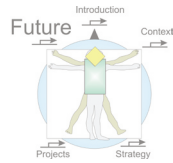
"I hope and expect that we will see a lot of activity in the area of 3Rs development during the next five years. The promotion of alternatives is an ongoing task of the highest importance, and through the EPAA we have a unique opportunity to push forward innovative ways to replace, reduce and refine animal use, for the dual benefit of the animals and the consumer, who will profit from better protection that modern toxicological testing methods promise."

J. Potočnik, European Commissioner for Environment. In: EPAA Newsletter, March 2011.

5.1 Introduction

It is the ambition of the **SEURAT-1** Research Initiative to contribute to the establishment of a new paradigm in toxicology, which will be based on a better understanding of toxic mechanisms rather than the description of phenomena. It is time for a new definition of "adversity" in toxicology at the cellular and molecular level, leading to pathway-based human safety assessment. Under this perspective, the **SEURAT-1** approach is to establish a complex system consisting of stable human cell lines and organ-simulating devices in combination with computational chemistry, systems biology and sophisticated modelling and estimation techniques. For the implementation of this concept, emphasis will be put on the reliability and reproducibility of all components necessary. However, taking into account the complexity of the problems to be solved and the broadness of 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 five years.

Consequently, the **SEURAT-1** Research Initiative will start to anticipate its second phase already at the beginning of the research programme. This chapter will provide the first steps with respect to future activities, and three components will be addressed here: (i) the realistic evaluation about what is achievable within the five years of the **SEURAT-1** Research Initiative; (ii) the identification of complementary research activities in Europe as well as on the international level (iii) the initiation of international co-operations. The aim is to establish international co-operation as close as possible over the course of **SEURAT-1**, and to advance scientific progress in this very dynamic 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.



5.2 The SEURAT-1 Research Initiative: Towards a more Realistic Integrated *In Vitro* Strategy for Repeated Dose Toxicity Testing of Cosmetic Products and their Ingredients

Vera Rogiers, Marleen Pauwels, Mathieu Vinken, Tamara Vanhaecke

5.2.1 Introduction

During the development of a cosmetic product considerable scientific input is required. A plethora of research groups involved in this process is not only concerned with searching for innovative ingredients and their efficacy assessment but also with novel applications and the formulation of the final product. Of paramount importance is the safety for human health and the environment of the final product and its constituents. Indeed, cosmetic products are not allowed to be placed on the EU market unless their safety for the consumer has been scientifically proven (*Anonymous, 1976*).

Whereas the safety standards for cosmetics vary between different parts of the world, it is generally accepted that Europe has laid down a stringent regulatory framework to ensure cosmetic safety. It is, however, mainly based on the use of experimental animals.

During the past decade, it has become clear that in the EU the political will exists to minimise the number of animals involved in safety testing of chemical substances in general. The driving force is not only ethical concern for the use of animals, but also recognition of important scientific progress, notably in molecular biology and biotechnology, which could obviate their usage. Thus, the progress in stem cell research, “omics” technology, systems biology, computational sciences and artificial organ design facilitates the development and establishment of 3R-alternative methods that could provide a higher or at least the same level of safety as obtained through existing animal-based methods. This prospect was advocated by the epaa (European Partnership for Alternative Approaches to Animal Testing), recommending the use of novel and increasingly available technologies, to address concerns with current animal-based safety models and to move science towards mechanistically-driven prediction rather than to simple observation (*epaa, 2008*).

5.2.2 Actual Safety Evaluation of Chemicals in the EU

The Cosmetics Directive 76/768/EEC

The basis of the regulatory framework for placing cosmetic products on the EU market lies in the Cosmetics Directive 76/768/EEC (*Anonymous, 1976*). This Directive has been amended several times, the most important ones being the so-called 6th (*Anonymous, 1993*) and 7th Amendment (*Anonymous, 2003*), which brought in a number of important alterations. In particular, the 7th Amendment introduced major changes with respect to non-animal testing (*Rogiers and Pauwels, 2008*).

Key points of the actual EU legislation can therefore be summarised as follows:

- A cosmetic product must be safe for the consumer (art. 2).
- The overall safety of the end product is based upon safe ingredients [art. 7 a(d)].
- The ultimate responsibility for guaranteeing safety lies with the manufacturer, first EU importer or marketer (art.2).
- The safety of cosmetics must be guaranteed without animal testing and stringent testing and marketing bans have hereby been firmly put in place.

Directive 76/768/EEC foresees a phasing-out of animal testing for cosmetics in the EU. A European ban on animal testing of finished cosmetic products has been in force since September 2004, and a testing ban on ingredients or combinations of these since 11 March 2009. A marketing ban prohibits cosmetics and their ingredients, which have been tested on animals after that date to be introduced on the EU market, irrespective of the origin of these products. An exception exists for the more complex endpoints including repeated dose toxicity, reproductive toxicity and toxicokinetics, for which the deadline of the marketing ban is extended to 11 March 2013. In this context, repeated dose toxicity includes here subacute toxicity (28 days), subchronic toxicity (90 days), chronic toxicity (> 12 months), carcinogenicity (2 years) and skin sensitisation.

The Cosmetics Directive 76/768/EEC will soon be replaced by a Regulation (*Anonymous, 2009*) but in principle no major changes are foreseen with respect to the phasing-out of animal testing for cosmetics products and their ingredients.

Safety evaluation of cosmetic ingredients

As outlined above, safety of cosmetics in the EU largely relies on yhe use of safe ingredients. To this end, Dir. 76/768/EEC foresees that the ingredients of a finished cosmetic product need

to be examined for their individual chemical structures, their toxicological profiles and their levels of exposure in order to result in safe use.

According to the Scientific Committee on Consumer Safety's Notes of Guidance (*SCCS, 2011*), the risk assessment process of cosmetic ingredients is mainly based upon the same principles and practices as usually applied in risk assessment for other product types including pharmaceuticals, plant protection products and food additives. However, in the cosmetic world it is generally preferred to use the expression "safety evaluation", which in fact has the same meaning as "risk assessment". Quantitative risk assessment is based upon risk characterization, which is composed of three pillars being hazard identification, dose-response assessment and exposure assessment. Risk characterization results in a number of measures regarding risk management and subsequent risk communication to ensure safe use by the consumer. Safety evaluation is an objective quantification, carried out by experts, of the probabilities and consequences of adverse effects. In fact, this comes down to looking for a "safe dose" for every ingredient of a cosmetic product under the intended use conditions.

In the EU, two distinct channels are operative for the safety evaluation of cosmetic ingredients (*Figure 5.1*). These include:

- The safety evaluation of the ingredients present on the Directive's Annexes IV, VI, VII, III or II, being colorants, preservatives, UV-filters, substances which are only allowed under restricted application and/or concentration, and forbidden substances, respectively, is carried out at the Commission level by the SCCS, (previously called Scientific Committee on Consumer Products, SCCP, or Scientific Committee on Consumer Non-Food Products, SCCNFP). It is anticipated that these chemical substances raise higher concern with respect to human health.

- The safety evaluation of all ingredients present in finished cosmetic products is the responsibility of the industry involved. For every cosmetic product a technical information file (TIF) or product information requirement (PIR), commonly referred to as "dossier", has to be prepared before the product may be introduced on the EU market. Safety evaluation is done by an independent safety assessor who reports back to the responsible industry.

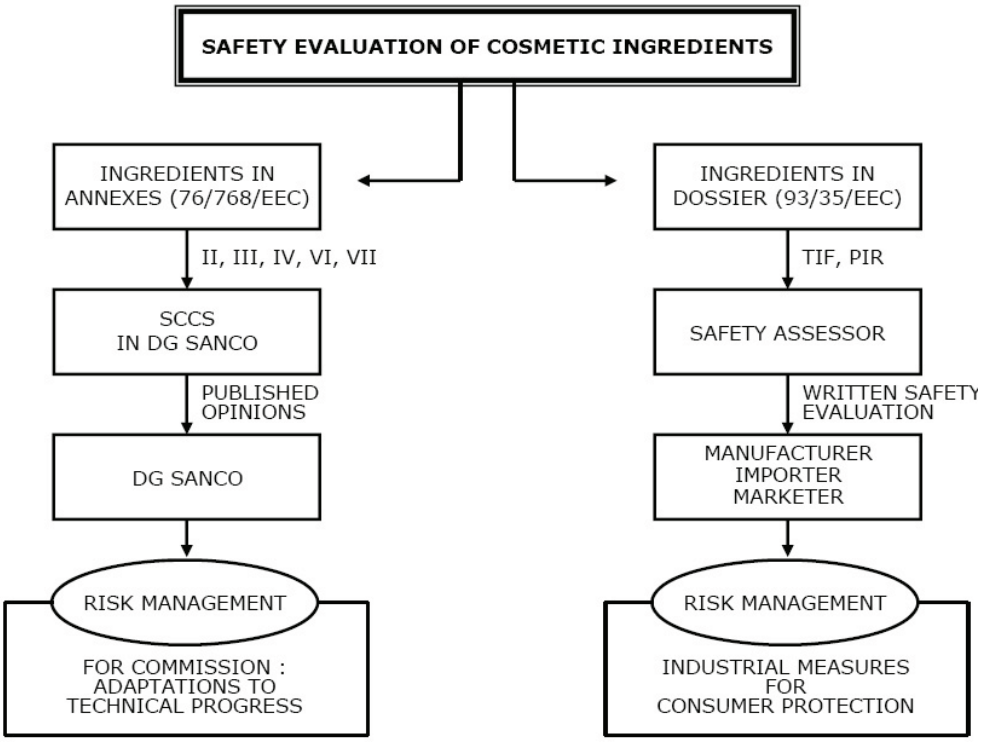


Figure 5.1 *Existing ways in the safety evaluation of cosmetic ingredients in the EU (SCCS, 2011). II: forbidden substances; III: forbidden substances but with limited exceptions for restricted concentrations and/or applicability domains; IV: allowed colorants; VI: allowed preservatives; VII: allowed UV-filters; DG: Directorate General; SCCS: Scientific Committee for Consumer Safety; TIF: Technical Information File; PIR: Product Information Requirement.*

In this way risk management and risk communication are taken care of at two distinct levels. In the former case it may result in legislative changes, namely adaptations to chemical progress (ATPs). In the latter case, industrial measures are taken for consumer protection. For more information, we refer to the SCCS's Notes of Guidance (*SCCS, 2011*).

Alternative methods in toxicity testing of cosmetics and their ingredients

In the safety evaluation of cosmetics, the first phase involves hazard identification. Usually, a vast list of measurements and tests are carried out on the cosmetic ingredients, in particular on substances taken up in the Annexes of Directive 76/768/EEC (*Table 5.1*).

Table 5.1 *Hazard identification usually required for cosmetic ingredients present on the Cosmetics Directive Annexes (SCCS, 2011).*

Hazard identification required
Acute toxicity (if available)
Irritation and corrosivity
Skin sensitisation*
Dermal / percutaneous absorption
Repeated dose toxicity*
Mutagenicity / genotoxicity
Carcinogenicity*
Reproductive toxicity*
Toxicokinetics*
Photo-induced toxicity
Human data

* Long-term test that today can be carried out outside the EU using animals and for which marketing within the EU still is possible.

Traditionally, experimental animals are used for most of these tests. However, compared to other types of chemical substances, the overall number of animals, mainly rodents, consumed for the cosmetics sector is relatively limited and amounts to 0.2% of the total number of laboratory animals involved per year for experimental and other scientific purposes in the Member States of the EU (*European Commission, 2010*). Nevertheless, as a consequence of the 7th Amendment, animal testing is currently drastically restricted and the ultimate goal is that only so-called “Replacement” alternatives being fully devoid of animal use, could be applied in the near future for safety testing of cosmetics. Indeed, the 3Rs principle (Refinement, Reduction, Replacement) of *Russell and Burch (1959)*, present in other areas of EU legislation, including REACH (Registration, Evaluation, Authorisation and restriction of Chemicals) (*Anonymous, 2006*), is for cosmetics restricted to only one “R”.

For acute systemic and local toxicity, a number of alternative methods are available, albeit not always animal-free. However, for long-term systemic toxicity testing the situation is becoming quite dramatic (*SCCS, 2011*). Indeed, although a number of long-term tests (indicated with an * in the list given above) still can be carried out using animals outside the EU and be marketed within Europe until March 2013, no alternative testing platform is presently in place to test

such endpoints and in particular subchronic and chronic toxicity. It is clear that replacing repeated dose toxicity testing in animals by a suitable animal-free alternatives remains a real challenge. The commonly used one-by-one replacement approach, successful to a certain extent for acute and local toxicity, is largely insufficient to cover the complexity of an *in vivo* whole body experiment, in which interactions occur between the different organs, tissues and cells and where different pathways are involved in the different compartments in the organism (*SCCS, 2011; Adler et al., 2011; Vanhaecke et al., 2011; 2009*). Yet, the **SEURAT-1** Research Initiative, starting in January 2011 and representing 50 million euro financial support by a joint effort of the European Cosmetics Association (Colipa) and the European Commission, intends to change this situation. To achieve this ambitious goal, coordinators and partners of the 7 projects involved in the so-called "building blocks" in this cluster should closely collaborate to ultimately replace animal-based repeated dose toxicity. In *Table 5.2*, the 7 projects with their acronym and main goal are presented. Indeed, a number of results that are essential for a positive outcome of the entire project, need to be produced in a coordinated and well-orchestrated way. In the next parts, it will be described how risk assessment is currently done, based on *in vivo* data in order to demonstrate what *in vitro* results are essentially needed from each project to come to a reasonable "replacement strategy" for repeated dose toxicity testing.

Table 5.2 *Projects of the* ***SEURAT-1*** *Research Initiative forming the "building blocks" of the integrated in vitro strategy for repeated dose toxicity testing of cosmetic products and their ingredients.*

Acronym	Content of building block
SCR&Tox	stem cell technology
HeMiBio	liver organ-simulating cellular devices
NOTOX	Molecular pathways
DETECTIVE	clinically relevant biomarkers
COSMOS	*in silico* tools, physiologically-based pharmacokinetic computational modeling and QSAR
ToxBank	Framework for data management and modelling, compound database and repository, reference resource for cells and tissues
COACH	overall facilitating and coordinating project

5.2.3 Proposed *in vitro* Strategy versus Current *in vivo* Methodology for Repeated Dose Toxicity Testing

Current *in vivo* methodology

Within actual SCCS dossiers, the most frequently occurring long-term toxicity study is the subchronic toxicity study of 90 days (*Rogiers and Pauwels, 2008*). It is an *in vivo* assay which consumes 80 animals per test and in which the animals usually rodents are daily orally exposed to the compound under investigation. Although most cosmetic products are applied topically by consumers, oral administration tests are carried out to represent a worst case scenario. In fact, most *in vivo* data available for cosmetic ingredients are obtained through that route (*Rogiers and Pauwels, 2008*). Throughout the testing period of 90 days, multiple observations and measurements are carried out on a variety of tissues, organs (30 in total) and body fluids in order to ultimately establish a so-called "No Observable Adverse Effect Level" (NOAEL). This value is one of the key tools in quantitative risk assessment (*Figure 5.2*). It is the dosage in the experimental animals that does not provoke adverse effects.

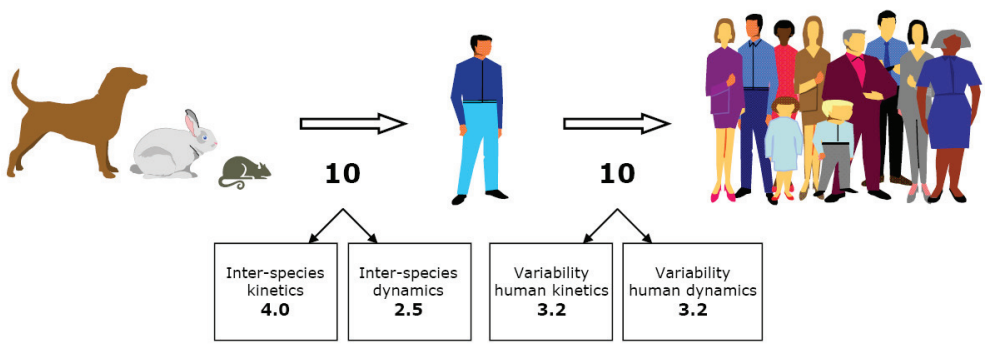


Figure 5.2 *Schematic representation of the extrapolation from animal to man, taking into account kinetic and dynamic inter- and intraspecies extrapolation. Based on Renwick (1998).*

This value is compared with the systemic exposure dosage (SED) and, in compliance with the World Health Organisation (WHO), an uncertainty factor of at least 100 should be present, counting 10 for the kinetic and dynamic interspecies variability (extrapolation from animal to man) and 10 for the intraspecies differences (extrapolation between human beings). For cosmetics, the uncertainty factor is traditionally called the "margin of safety" or "MoS":

$$MoS = \frac{NOAL}{SED}$$

In *Figure 5.3*, the entire process of *in vivo* risk assessment is summarised, starting with the collection of all possible information on the compound, including physico-chemical data, (quantitative) structure activity relationship [(Q)SAR] data, existing *in vivo* and *in vitro* data on related substances and, if available, also clinical and epidemiological data. For the sake of clarity, the *in vitro* strategy proposed further onwards will refer to the current *in vivo* approach as presented in *Figure 5.3*.

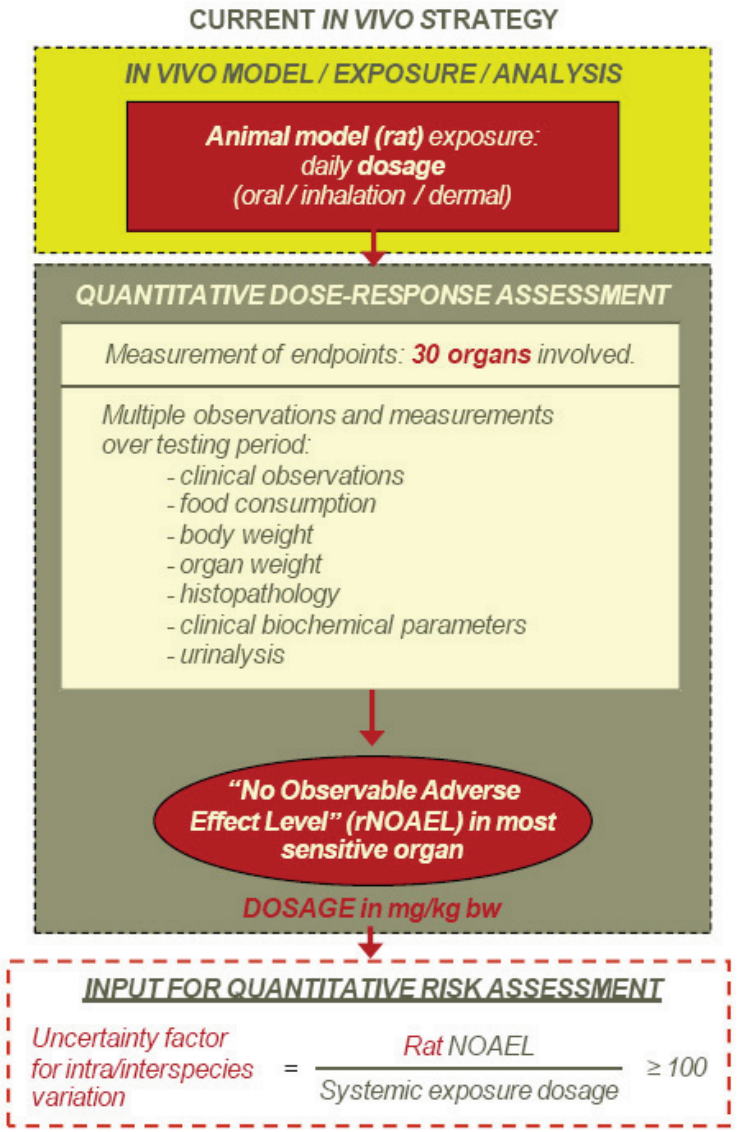


Figure 5.3 *Summary of the current in vivo risk assessment process based on in vivo repeated dose toxicity testing. Rat NOAEL = No Observable Adverse Effect Level in rat.*

Setting up an integrated approach for the *in vitro* assessment of repeated dose toxicity

The liver is one of the most important target organs for chemical-induced liver toxicity, as well for pharmaceuticals (*Batt and Ferrari, 1995; Xu et al., 2004; Russmann et al., 2009*) as for cosmetics (*Pauwels et al., 2010*). Furthermore, the liver and, in particular the hepatocyte constitutes the main site of xenobiotic biotransformation in the organism and is as such taken up in all **SEURAT-1** research projects. It seems therefore evident to set up a proof of principle related to liver toxicity.

The *in vitro* strategy proposed here for addressing liver toxicity in repeated dose toxicity testing is based on three essential pillars:

1. Identification of human relevant parameters and selection of reference compounds

It is of crucial importance to select from the scientific literature, existing databanks, and through expert experience a set of reference compounds showing clear-cut hepatotoxic effects (ToxBank) and measurable clinically relevant biomarkers (DETECTIVE) and molecular pathways (NOTOX) indicative for human long-term systemic liver toxicity. The latter is possible via the integration of "-omics" and functional analysis (*Figure 5.4*).

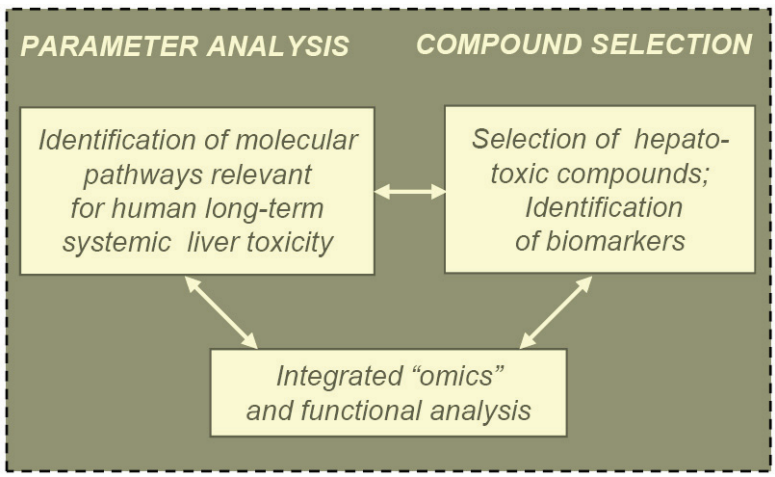


Figure 5.4 *Identification of human relevant parameters and selection of reference compounds: one of the essential pillars in the proposed in vitro strategy.*

2. Renewable metabolic competent human hepatocyte-based cell systems

Sufficient numbers of metabolically competent human hepatocytes are required for routine industrial toxicity testing procedures. At present, the most obvious way to achieve this goal is through stem cell technology (*SCR&Tox*). The so-obtained human hepatocytes could then be used as a "stand-alone" *in vitro* model, but they also represent the major cell type for

feeding into a liver organ-simulating device (*HeMiBio*) where special attention is paid to the "natural" micro-environment of the hepatocytes in the liver. It is obvious that these stem cell-derived hepatocytes and/or liver organ systems have to undergo thorough standardisation before they can be implemented in an integrated repeated dose toxicity strategy. The following step then consists of measuring the predefined parameters after daily exposure to different concentrations of the hepatotoxic reference compounds for a period of at least 14 days in order to generate appropriate dose-response curves to establish an *in vitro* "human No Observable Adverse Effect Concentration" (hNOAEC; *Figure 5.5*).

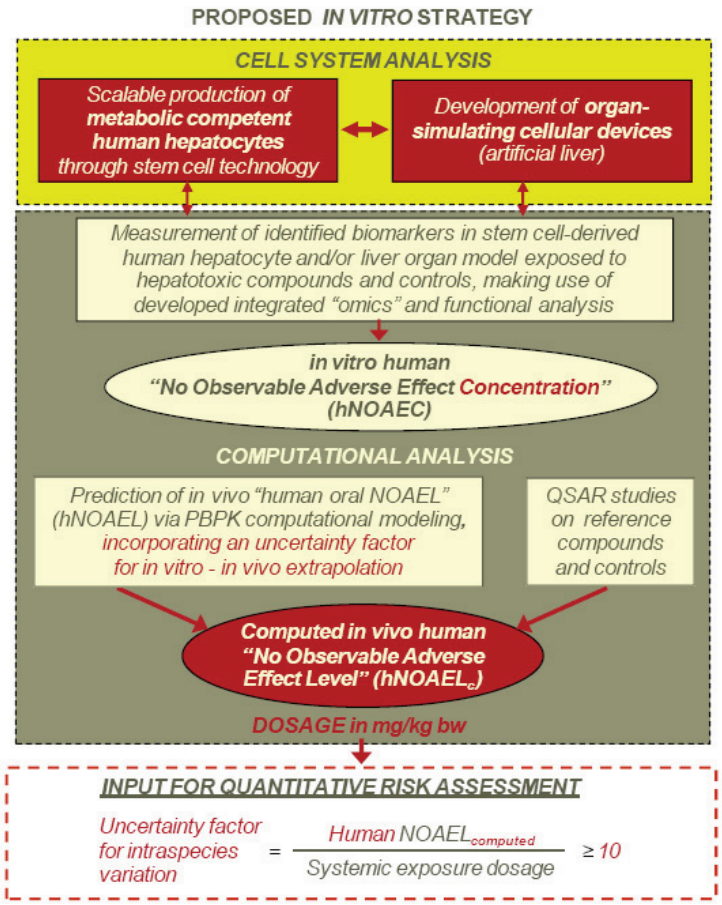


Figure 5.5 *Proposal of an integrated approach for the in vitro assessment of repeated dose liver toxicity. hNOAEC = human No Observable Adverse Effect Concentration; hNOAEL = human oral No Observable Adverse Effect Level; hNOAELc = computed human No Observable Adverse Effect Level; NOAEL = No Observable Adverse Effect Level; QSAR = Quantitative Structure Activity Relationship; PBPK modeling = Physiologically-Based PharmacoKinetic modelling.*

3. Computational modeling

In silico tools are needed to estimate the *in vivo* "human oral No Observable Adverse Effect Level" (hNOAELc; COSMOS). This can be done by applying physiologically-based pharmacokinetic (PBPK) computational modeling to the hNOAEC value measured in the above cell/organ system(s) and by taking into account an appropriate uncertainty factor for the *in vitro – in vivo* extrapolation. Simultaneously, structure activity relationship (QSAR) studies should be performed on the selected set of hepatotoxic compounds (*Figure 5.5*).

Whenever scientifically sound results are obtained following the above described procedure, the computed *in vivo* hNOAELc could be used as a quantitative risk assessment determinant. As the *in vitro* experiments are carried out on human cells, interspecies variation disappears as a factor of concern. Assuming that the PBPK modeling already took into account the *in vitro-in vivo* extrapolation, the ratio of the obtained hNOAELc and the systemic exposure dosage (= the intra-species uncertainty factor), needs to be at least 10 in order to consider the compound as safe with regard to its hepatotoxic effects. Of course, additional uncertainty factors can be introduced during the entire process.

It will be of utmost importance to check the *in vivo* relevance of the proposed *in vitro* strategy with reference compounds. Therefore, it is key that the compounds selected are well-known substances causing so-called drug-induced liver injury (DILI) in humans and that reliable *in vivo* data are available for these compounds. The same compounds should be consistently used in the different research projects of the **SEURAT-1** Research Initiative. This challenging task will be coordinated by ToxBank.

The *in vitro* strategy proposed here for repeated dose toxicity testing (as summarised in *Figure 5.5*) has a lot of similarity with the currently used *in vivo* strategy shown in *Figure 5.3*. It is therefore believed that this pragmatic way of working could ultimately result in an acceptable procedure for regulatory acceptance. It is of course of primary importance that a high concordance of the generated *in vitro* results can be shown with the existing *in vivo* human data for a series of reference compounds. The latter should be made available at the start of the **SEURAT-1** Research Initiative by the pharmaceutical and cosmetic industry.

5.2.4 Conclusion and Perspectives

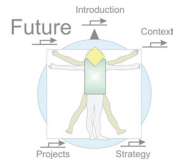
In the best case scenario, the expected and realistic outcome of the different projects in the **SEURAT-1** Research Initiative would be the establishment of (i) functional (stem cell-derived) human liver cells/cellular devices (*SCR&Tox*, *HeMiBio*), (ii) clinically relevant human endpoints (DETECTIVE, NOTOX) and (iii) practically applicable PBPK models (COSMOS).

If reference hepatotoxic compounds with reliable human liver toxicological data, e.g.

pharmaceuticals and cosmetic ingredients, are being applied by all groups involved, the i*n vivo* relevance of the *in vitro* strategy can be verified for these reference compounds, with liver as the target organ. If encouraging results are obtained, analogous schemes could be worked out for other important target organs for toxicity, including kidney, lung and heart. Finally, a selection needs to be done of the major target organ involved in the toxicological events observed and the lowest computed hNOAEL chosen. Further, ADME (Absorption, Distribution, Metabolism, Excretion) modeling optimization probably will remain necessary, in particular for the distribution and excretion parameters. A further challenge lies in the coverage of the whole body complexity and the manifold interactions between cells, tissues and organs. It is therefore clear that a considerable amount of work is being faced after successful completion of the *in vitro* approach for liver.

Acknowledgements

The authors are involved in the EU projects *HeMiBio* (FP7 Project Reference 266777) and DETECTIVE (FP7 Project Reference 266838).



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5.3 Related International Activities

The COACH Team

The following sections provide an overview about ongoing international activities, which are related to the objectives of the **SEURAT-1** Research Initiatives. The descriptions were kept very brief and were in parts directly taken from published corresponding project descriptions. These used sources for information are given at the end of each project description (in general, this refers to a public web-page).

5.3.1 European Activities

EU FP7: 7th Framework Programme of the European Union represented by the European Commission

The 7th Framework Programme for Research and Technological Development (FP7) will run from 2007 until 2013 as the main financial instrument for research funding in Europe. As with the other EU Framework Programmes, FP7 is the results of a proposal by the European Commission and adoption by the Council and the European Parliament. The core of the funding scheme is the Cooperation Programme, which primarily promotes international scientific collaboration carried out by ten thematic areas, of which one is the HEALTH Theme.

The overall objective of the HEALTH Theme under FP7 is to improve the health of European citizens and to boost the competitiveness of health-related industries and businesses, as well as address global health issues. The 2012 calls for proposals related to the HEALTH Theme are expected to be published in July 2011, covering four main research areas, which are (1) Biotechnology, Generic Tools and Medical Technologies for Human Health (aim: development and validation of the necessary tools and technologies that will enable the production of new knowledge and its translation into practical applications in the area of health and medicine), (2) Translating Research for Human Health (aim: increasing knowledge about biological processes and mechanisms involved in normal health and in specific disease situations, to transpose this knowledge into clinical applications including disease control and treatment, and to ensure that clinical (including epidemiological) data guide further research), (3) Optimising the Delivery of Health Care to European Citizens (aim: improving the necessary basis both for informed policy decisions on health systems and for more effective and efficient evidence–based strategies of health promotion, disease prevention, diagnosis and therapy), (4) Other Actions across the HEALTH Theme (aim: to contribute to the implementation of the

Framework Programme and the preparation of future European Union (Community) research and technological development policy). Further details about the respective calls for proposals can be found on the related homepage of the European Commission.

More information: http://cordis.europa.eu/fp7/health/

OSIRIS: Optimised Strategies for Risk Assessment of Industrial Chemicals trough Integration of Non-Test and Test Information

OSIRIS is an Integrated Project funded under the European Commissions 6th Framework Programme (FP6) that started in 2007 and will run until September 2011. The consortium comprises 31 partners from 14 different countries of the European Union. The aim is the development of new Integrated Testing Strategies (ITS) in toxicity testing in the context of the EU regulation for chemicals REACH.

Driven by the principles of REACH to evaluate chemicals in a more risk-driven, context-specific and substance-tailored way, OSIRIS aims to strengthen the use of alternative methods such as exposure-based waiving, chemical and biological read-across, QSARs (qualitative and quantitative structure-activity relationships), *in vitro* tests as well as optimisation of *in vivo* tests. The Integrated Project is organised in five interlinked research pillars, which represent (1) the Chemical Domain, (2) the Biological Domain, (3) Exposure, (4) Integration Strategies and Tools and (5) Case Studies.

Scientific Coordinator: Gerrit Schüürmann (Helmholtz Centre for Environmental Research – UFZ, Germany)

More information: http://www.osiris-reach.eu/

IMI: Innovative Medicines Initiative

IMI is Europe's largest public and private sector collaboration between public authorities, biopharmaceutical companies, patient organisations, universities and other organizations. The development of IMI can be traced back to the European Technology Platform on Innovative Medicines that was launched by the European Commission and the European Federation of Pharmaceutical Industries and Associations (EFPIA) under the 6th Framework Programme for Research in 2004. Based on this successful establishment the European Commission and EFPIA have decided to create Joint Technology Initiatives (JTIs) as European public-private partnerships in research under FP7. The Innovative Medicines Initiative Joint Undertaking (IMI JU) is one of the first of these unique partnerships. The primary goal is to improve the drug development process by supporting a more efficient discovery and development of better and safer medicines for patients. This should improve the competitiveness of the European

Union in the biomedical and pharmaceutical sector by pooling competencies and resources from different domains.

The research projects that are selected for funding by IMI through open calls for proposals have to follow the four strategic areas (the so-called Four Pillars) that were identified in the Strategic Research Agenda: (1) Predictivity of Safety Evaluation, (2) Predictivity of Efficacy Evaluation, (3) Knowledge Management, and (4) Education and Training. At present, there are 23 on-going IMI research projects covering a diverse set of thematic areas, such as safety of drugs (e.g. based on identification of biomarkers, MARCAR project; development of novel software tools to better predict the safety and the side-effects of new candidate medicines for patients, eTOX project), different diseases (Diabetes, neurodegenerative diseases, Depression and Schizophrenia) and Training Programmes. Furthermore, there are 8 new projects resulting from the 2nd and 3rd Call for Proposals dealing with topics such as oncology, inflammation and infection, drug vaccine safety, tuberculosis, autism, but also knowledge management, and public awareness.

Executive Director: Michel Goldman (Université Libre de Bruxelles, Belgium)

More information: http://www.imi.europa.eu/

AXLR8: Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development

AXLR8 is a coordination action funded within the European Commissions 7th Framework Programme under the HEALTH Theme. It has been established as a focal point for dialogue, collaboration and coordination among 3Rs ("Replacement, Reduction and Refinement of animal test") research projects at national, European and international levels. The purpose of the project is to monitor the EU FP6/FP7-funded 3Rs research activities and related international initiatives with the aim of accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment. AXLR8 will facilitate the swift progress to more mechanistic cell- and computer-based techniques. This overarching goal is subdivided in different specific objectives: (1) Monitoring activities of new DG-RTD-funded FP6/FP7 3Rs research consortia and preparation, publication and dissemination of progress reports on annual basis. (2) Organisation of annual workshops to monitor research progress, identify gaps and needs in the FP6/FP7 programme on alternative testing strategies. (3) Promoting establishment of a Scientific Panel (SP) as a platform for information exchange comprising representatives of EU-funded 3Rs research projects together with independent experts from the EU corporate, governmental and academic sectors. (4) Gain of stakeholders and awareness of the public regarding the outcomes of AXLR8 scientific workshops and safety assessment. (5) Promotion of viable and ongoing engagement by European regulatory authorities in order to streamline

regulatory acceptance procedures and to provide for the uptake of validated 3Rs methods, including a smooth transition to 21st century systems, as they become available.

Scientific Coordinator: Horst Spielmann (Freie Universität Berlin, Germany)

More information: http://axlr8.eu

ChemScreen: Chemical substance *in vitro* / *in silico* screening system to predict human- and ecotoxicological effects

ChemScreen is a collaborative project, which is funded within the European Commissions 7th Framework Programme (FP7) under the Environment programme. The project started in 2010 and will run for 4 years. ChemScreen is a sister project of the US Environmental Protection Agency's National Center for Computational Toxicology (NCCT/STAR centre) and is therefore strongly linked to related projects in North America (Toxcast, Tox21; see project descriptions below). Nine project partners from five countries of the European Union are working together in ChemScreen with the overall goal to develop innovative, animal free screening methods for the assessment of toxicological and eco-toxicological effects of chemicals in the field of reproductive toxicity.

Scientific Coordinator: Bart van der Burg (BioDetection Systems BV, Amsterdam, The Netherlands)

More information: http://chemscreen.eu/

Predict-IV: Profiling the toxicity of new drugs: a non animal-based approach integrating toxicodynamics and biokinetics

Predict-IV is a collaborative large-scale Integrated Project, which is funded within the European Commissions 7th Framework Programme (FP7) under the HEALTH Theme. Predict-IV started in 2008 and will run until 2013. Overall, 21 European participants of different scientific sectors (academia, industry) are working in this project. The overall goal is the development of new strategies for improved assessment of drug safety in the early stage of development and a late discovery phase.

The project is motivated by the existing deficit in view of preclinical toxicity testing approaches, which can be explained by both the lack of therapeutic efficiency and unpredicted toxicity in animals and humans. New acquisitions in tissue and bioreactor technologies, molecular biology, toxicity modelling and bioinformatics are integrated in Predict-IV to improve and optimise cell culture systems for toxicity testing. Predict-VI will form a combination of classical *in vitro* toxicology and recent technologies, profiling and modelling tools in a system biological approach. High quality standards on modelling and biostatical analysis will be used for analysis, evaluation and integration of data from *in vitro* experiments. Additionally, Predict-

IV will dispose advances in “omics” technologies and high-content imaging and therefore increase the probability for the early identification of toxic effects of pharmaceuticals.

Scientific Coordinator: Wolfgang Dekant (Universität Würzburg, Germany)

More information: http://www.predict-iv.toxi.uni-wuerzburg.de/

CancerSys: Mathematical modelling of beta-catenin and ras signalling in liver and its impact on proliferation, tissue organisation and formation of hepatocellular carcinomas

CancerSys is funded within the European Commissions 7th Framework Programme (FP7) under the HEALTH Theme. The project started in 2008 and will run until November 2011. The consortium comprises 8 European research institutions. CancerSys is based on the foundation of the HepatoSys consortium launched in 2004 as the first interdisciplinary network for research on hepatocytes in the field of Systems Biology, which represents a combination of quantitative methods used in molecular biology completed by theoretical background of mathematics, informatics and system science. The goal of CancerSys is to establish a multi-scale model for two major signalling pathways involved in the formation of hepatocellular carcinoma.

CancerSys will elucidate the impact of the beta-catenin- and the ras-signalling pathway on cell proliferation, tissue organisation and formation of hepatocellular carcinoma. These two signalling pathways play an important role in the proliferation regulation in hepatocytes and are therefore potentially involved in gene activity alteration during cancer development. The identification of ras- or beta-catenin- related genes deregulated during carcinogenesis will allow more accurate predictions of effective intervention strategies in the development of new approaches concerning therapy of hepatocellular carcinoma.

Scientific Coordinator: Jan G. Hengstler (Leibniz Research Centre for Working Environment and Human Factors, Dortmund, Germany)

More information: http://www.ifado.de/cancersys/index.html

ESNATS: Embryonic stem cell-based novel alternative testing strategies

ESNATS is also funded within the European Commissions 7th Framework Programme (FP7) under the HEALTH Theme. It started in 2008 and will run until 2013. All in all, 27 European participants are involved in ESNATS. This project demonstrates a new type of platform for toxicity testing using the different advantages of embryonic stem cells (ESCs) including especially human ESCs. Using these cell-types characterised by their self-renewal capacity, their pluripotency and the impact of ES-derived somatic and murine cells, the project aims

to achieve three overall objectives: to accelerate drug development, to reduce related Research and Development costs and to propose a powerful alternative to animal tests. The achievement of these key objectives implies the examination of different ethical issues involved in the ESNATS scientific programme when working with ESCs. For this reason, the work of the ESNATS consortium is advised by the ethical organisation Edinethics Ltd in order to comply with the strict requirements specified by the European Commission for FP7 research programmes.

ESNATS is subdivided into four key research areas, covering the following complementary scientific aspects: (1) The sub-project entitled "Reproductive Toxicity" will investigate the hazard of compounds to the reproductive cycle, i.e. impact on fertilisation, differentiation into gametes (male fertility), and early embryonic development; (2) the sub-project "Neurotoxicity" deals with the effects of compounds on neuronal development and viability (functionality); (3) the sub-project "ESC-based toxicogenomics and toxicoproteomics" focuses on the influence of compounds on gene expression and proteomics using *in vitro* test systems suitable for high-throughput methods; (4) the sub-project "Metabolism, Toxicokinetics and Modelling" concentrates on the development of physiologically based pharmacokinetic (PBPK) models using *in vitro* data.

Scientific Coordinator: Jürgen Hescheler (Universität Köln, Germany)

More information: http://www.esnats.eu

OpenTox: Promotion, development, acceptance and implementation of QSARs (quantitative structure-activity relationships) for toxicology

OpenTox is another project funded within the European Commissions 7th Framework Programme (FP7) under the HEALTH Theme. It terminates in 2011 after 36 months duration. The overall goal of this collaborative project with 11 European participants was to develop a predictive toxicology framework with a unified access to toxicological data, (Q)SAR models and supporting information.

The participants created OpenTox as a community framework under the guidance of different experts of the industry and regulatory sector. This framework is based on the combination of multiple web services and allows open access to toxicological relevant information sources including data, ontologies and models. Different international authorities (e.g. ECB, ECVAM, US FDA, US EPA) and industrial enterprises supported these activities as members of the OpenTox advisory board. In general, the project gives users the opportunity to profit from various data sources (public and confidential), the generation and validation of (Q)SAR models, and the integration of (Q)SAR algorithms assembled in libraries and validation routines.

Scientific Coordinator: Barry Hardy (Douglas Connect, Switzerland)

More information: http://www.opentox.org

Sens-it-iv: Novel testing strategies for *in vitro* assessment of allergens

Sens-it-iv was funded within the European Commissions 6th Framework Programme (FP6). It terminated in 2010 after 60 months duration. The overall goal of this integrated project with 28 European participants from academia as well as from industry was to develop non-animal tests and testing strategies to assess allergenic potential of chemicals. The project ultimately aimed on the establishment of innovative *in vitro* assays ready for pre-validation by ECVAM (the European Center for Validation of Alternative Methods). This is seen in relation to the use of safe ingredients by the chemical, cosmetic and pharmaceutical industry.

The integrated project was organised in 10 work packages and the objectives were subdivided into scientific and technological ones. The work was organised in a Science Module and a Technology Module, respectively. The Science Module was active in the first 3 years of the project and developed fundamental understanding of how anticipated key players in skin sensitisation interact in undisturbed tissues, and how these interactions are affected by potential sensitisers. The Technology Module was active during the last 3 years (1 year overlap with the Science Module) with the task to further develop and refine assays to make them ready for pre-validation. The project identified seven potential *in vitro* assays that are currently being evaluated.

Scientific Coordinator: Erwin L. Roggen (Novozymes A/S, Denmark)

More information: http://www.sens-it-iv.eu/

Virtual Liver Network

The Virtual Liver Network funded by the Federal Ministry of Education and Research (BMBF) is a German, interdisciplinary research initiative that started in 2010 with a running time of five years. It is a successor of the HepatoSys network and focuses on the development of a multi-scale computer model of the liver. Over 70 research groups from 41 institutions across Germany work on the development of the Virtual Liver as a mathematical dynamic model integrating quantitative data from different scales (cellular, intercellular, lobular and organ level) for the exploration of human liver physiology, morphology and functionality.

The initiative is subdivided into nine different working areas addressing the following different aspects: (1) cellular metabolism, (2) integration of signalling pathways in hepatocellular responses (cellular signalling), (3) cross-linking signalling and metabolism/hepatocyte polarization, (4) communication of hepatocytes and non-parenchymal liver cells (cell-cell-communication), (5) liver lobule, (6) whole organ, (7) integrated modelling, (8) data management, and (9) clinical translation. The virtual representation of the human liver will allow a better understanding of liver functions and physiology in normal and diseased states. Furthermore, it will help to develop new therapies in a cost-effective way.

Scientific Administrator: Johannes Bausch (Universität Heidelberg, Germany)

More information: http://www.virtual-liver.de

ECVAM: European Centre for the Validation of Alternative Methods

ECVAM was established in 1992 at the European Commissions Joint Research Centre in Ispra, Italy, and is now part of the "*In Vitro* Methods Unit" (IVMU) of the Institute for Health and Consumer Protection (IHCP). Today, ECVAM provides the institutional basis to fulfil the requirements of the "Council Directive 86/609/EEC on the approximation of the laws, regulation and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes". Following this, ECVAM has the following two overarching objectives: (1) The promotion of the scientific and regulatory acceptance of non-animal tests through research, test development and validation and the establishment of a specialised database service; (2) the coordination of the independent evaluation of tests at the European level, so that chemicals and products of various kinds can be manufactured, transported and used more economically and more safely, whilst the current reliance on animal test procedures is progressively reduced. These goals are broken down into five tasks, which are (1) the coordination and promotion of the development and use of *in vitro* methods as alternatives to animal testing; (2) the coordination of the validation of alternative testing strategies at the level of the European Union, (3) the installation of an information exchange network focussing on new developments in alternative testing, (4) management of public databases and information systems, (5) the promotion of a dialogue between the involved key players in legislation, regulation and the different stakeholder groups (in academia, industry, consumer organisations, etc.).

More information: http://ecvam.jrc.ec.europa.eu/

SC4SM: Stem Cells for Safer Medicines

Stem Cells for Safer Medicines (SC4SM) was created as an independent, public-private collaboration between the UK government and three major international pharmaceutical companies: GlaxoSmithKline, AstraZeneca and Roche. This initiative is a direct follow-up of the UK Stem Cell Initiative. The ultimate goal of SC4SM is to establish a human stem cell bank comprising stably differentiated human cell lines for safety assessment of new medicines. For that, SC4SM also promotes the development of open protocols and standardised systems for the differentiation of stem cells into stable homogenous populations that are suitable for toxicology testing in high throughput platforms.

Cell types of interest for early toxicity screening in drug development comprise hepatocytes and cardiomyocytes. Currently, experts from industry and academia are engaged in a five-

year research programme focussing on the derivation and physiological characterization of cardiomyocyte cells (based on human stem cells). An independent Ethics Review Board supervises the work with human embryonic stem cells, providing a clear ethical framework in this sensitive area of research.

More Information: http://www.sc4sm.org/

CAAT-Europe: The Center Alternatives to Animal Testing - Europe

The Center for Alternatives to Animal Testing - Europe (CAAT-Europe) was founded in 2009 as a transatlantic joint venture between the Johns Hopkins Bloomberg School of Public Health, Baltimore, USA, and the University of Konstanz. All in all, the University of Konstanz has 20 years of experience in the field of alternatives to animal testing. CAAT-Europe critically evaluates *in vivo*, *in vitro* and *in silico* approaches. The aim is to bring together the industrial and academic sectors that are involved in the development of toxicity tests in order to serve the needs for establishing alternative methods.

The objectives of CAAT-Europe are (1) to bring together industry and academics to address the needs for human-relevant methods; (2) to make use of strategic funds to fill in the gaps in the development and implementation of alternative methods; (3) to coordinate workshops and information days in Europe on relevant developments in the area of alternatives and toxicology; (4) to develop strategic projects with sponsors to promote human science and “new toxicology”; (5) to develop a joint education programme between the Johns Hopkins University in Baltimore and the University of Konstanz; (6) to set up transatlantic consortia for international research projects on alternative methods; (7) to support ALTEX as the official journal of CAAT, the European Society for Alternatives to Animal Testing (EUSAAT), and the Transatlantic Think Tank for Toxicology (t4).

More information: http://cms.uni-konstanz.de/leist/caat-europe/

OECD Chemicals Testing - Guidelines

The OECD (Organization for Economic Co-Operation and Economic Development) Guidelines for the Testing of Chemicals are a collection of the most relevant internationally agreed testing methods used for safety assessment of chemicals. Different OECD working groups have been established, addressing the various approaches in the field of toxicity testing, which will be briefly discussed below.

The (Quantitative) Structure-Activity Relationship [(Q)SAR] Project was launched already in the early 1990s. This project has focused on the acceptance of (Q)SAR approaches for the evaluation of chemicals, focusing since 2004 particularly on the development of the OECD

(Q)SAR Toolbox. This software was created for governmental instances and stakeholders of chemical industry in order to bridge data the gaps in (eco)toxicology. Version 2 of the Toolbox was released in 2010. It can be used for the identification of potential toxic mechanisms of chemicals, including their metabolites. The Toolbox comprises all regulatory endpoints and contains "mechanistic profilers" for the identification of relevant mechanisms or modes-of-action.

The "Molecular Screening for Characterization Individual Chemicals and Chemical Categories Project" (Molecular Screening Project) was established in 2007 by the OECD in cooperation with the International Program on Chemical Safety (IPCS). The aim is to develop a strategy for prioritisation of chemicals to be further tested, based on the molecular properties that are linked to potential toxicity. High-throughput screening (HTS) using *in vitro* assays and selected chemicals is being applied for the evaluation of specific pathways.

The emerging area of toxicogenomics is also addressed by the OECD in collaboration with IPCS. The objectives are (1) to identify new biomarkers that are representative for specific pathways, and (2) to conduct surveys on existing toxicogenomic tools. The overall goal of these activities is the development of a strategy regarding the future application of toxicogenomics in the context of regulatory chemical safety assessment.

Contact: Patric Amcoff (OECD, Environment Directorate, Paris, France)

More information: http://www.oecd.org/env/testguidelines

EFSA: European Food Safety Authority

As a consequence of a series of food crises, the European Food Safety Authority (EFSA) was set up in 2002 by the European Union as an independent agency for risk assessment and risk communication, covering all aspects associated with the food chain. EFSA aims to provide appropriate, consistent, accurate and timely communications on food safety issues to all stakeholders and the public at large, based on the Authority's risk assessments and scientific expertise. Nearly 460 people are currently engaged at EFSA, working in the different food-related scientific fields, such as food and feed safety, nutrition, animal health and welfare, and plant protection. Giving independent scientific advice and assessing on all risks concerning the food chain, EFSA plays a major role in Europe`s food safety system.

More information: http://www.efsa.europa.eu/

SCCS: Scientific Committee on Consumer Safety

The SCCS is organised in the European Commissions Directorate General for Health and Consumers. It provides opinions on health and safety risks of non-food consumer products

(such as cosmetic products and their ingredients) and services (such as artificial sun tanning). The SCCP releases the “Notes of Guidance for the Testing of Cosmetic Ingredients and their safety Evaluation”, which is regularly updated according to progress made in science.

More information: http://ec.europa.eu/health/scientific_committees/consumer_safety/

5.3.2 International Activities

USA

Tox21: Toxicity 21

Tox21 is a joint initiative of the Environmental Protection Agency (EPA), the National Toxicology Program of the National Institute of Environmental Health Sciences, the National Institutes of Health (NIH) Chemical Genomics Center (NCGC), the National Human Genome Research Institute and the Food and Drug Administration (FDA). Tox21 is part of the EPA’s Computational Toxicology Research Program, which aims to provide high-throughput decision support tools for assessing chemical exposure, hazard, and risk. In this context, the overarching goal of Tox21 is to develop, validate and translate innovative chemical testing methods that characterise toxicity pathways. The knowledge about toxicity pathways will be used for prioritisation of chemicals that needs to be further tested and the development of innovative *in silico* methods.

The general approach is to screen a large number of chemicals (approximately 10.000) using high-throughput screening assays. Four different working groups were established within Tox21: (1) Assays / Pathways Group, which is responsible for the identification of key toxicity pathways/assays, the incorporation of hepatic metabolism into *in vitro* assays, and to establish methods that account for interactions between compounds, pathways, as well as between cells (cell-to-cell interactions); (2) Compounds Group, which is responsible for the establishment of a library containing the chemical structures of the 10.000 chemicals to be tested within Tox21, quality control issues, and the establishment of a library containing water soluble compounds and mixtures, respectively, to be tested in the future; (3) Bioinformatics Group, which is responsible for the data interpretation (response within and across assays and endpoints, respectively, response pattern and relationships with adverse outcomes in *in vivo* tests) and the accessibility of data by the public; and (4) Targeted Testing Group, which is responsible for the evaluation of the *in silico* methods and prioritisation schemes.

Scientific Coordinator: Robert Kavlock (Director of EPA’s National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/Tox21/

ToxCast™: Screening Chemicals to Predict Toxicity Faster and Better

The US Environmental Protection Agency (EPA) launched ToxCast in 2007 as the largest component of EPA`s Computational Toxicology Research Program for chemical screening. The National Centre for Computational Toxicology (NCCT) coordinates ToxCast. The aim is to develop a cost effective approach for prioritising the vast number of chemicals that still needs toxicity testing, and to predict potential toxicity of chemicals. ToxCast promotes the use of an extensive array of automated high throughput screening assays for the prediction of potential toxicity in order to generate profiles of environmental chemicals and to create initial prioritization models of *in vivo* toxicity.

ToxCast21 evaluates the impact of chemical exposure on biological processes in humans and the consequential health effects. It runs around 500 high-throughput assays for screening of approximately 1000 environmental chemicals for potential toxicity. The data are fed into the ToxCast database (ToxCastDB) and used for the elucidation of toxicity signatures. The predictive power of these toxicity signatures is currently being tested by another set of 1000 chemicals. The data are also available for the collaborators in the frame of Tox21.

Scientific Coordinator: Robert Kavlock (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/toxcast/

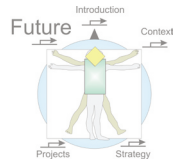
ToxRefDB: Toxicity Reference Database

The Toxicity Reference Database (ToxRefDB) is another project that is organised under the umbrella of the EPA's Computational Toxicology Research Program. It was developed by the National Centre of Computational Toxicology (NCCT) in collaboration with the EPA`s Office of Pesticide Programs (OPP). The aim is to set up a comprehensive database of *in vivo* animal toxicity studies. This will allow establishing links between toxicity pathways discovered in Tox21 and ToxCast (see above) and adverse outcomes *in vivo*.

The ToxRef database comprises several thousands of animal toxicity studies after testing hundreds of different chemical substances. ToxRefDB is the first database which makes chemical toxicity data accessible to the public offering pesticide registration toxicity data and data from (sub)chronic, cancer, reproductive and developmental studies. Furthermore, the database provides toxicity endpoints for the establishment of ToxCast predictive signatures.

Scientific Coordinator: Robert Kavlock (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/toxrefdb/



v-Liver™: The Virtual Liver Project

The Virtual Liver project was also established as a component of the EPA`s Computational Toxicology Research Program. The aim is to estimate the potential of chemicals to cause chronic diseases such as cancer by means of a large-scale computer model simulating dynamic liver processes.

The mechanistic understanding of chemical effect networks will serve as the basis for modelling the key molecular, cellular and circulatory systems in the human liver. Health effects of chemicals over time will be estimated by means of a cell-based tissue simulator. Furthermore, the risk of human cancer through ingestion (the oral pathway) will be quantitatively estimated for selected chemicals (integration of physiologically based pharmacokinetic modelling (PBPK), cellular systems and molecular networks to simulate *in vivo* effects of chemicals), and "Virtual Tissues" will be developed to evaluate the human health impact of chemicals using *in vitro* assays. Overall, the v-Liver project will predict chemical-induced effects on the human liver on the level of virtual hepatic lobules using three interconnected systems: (1) Simulation of micro-circulation and estimation of microdosimetry by using a vascular model network and *in vitro* data, (2) simulation of key molecular events involved in determining phenotypic state of cells by means of *in vitro* data, (3) simulation of the tissue response through a cellular systems model representing the complex interplay between hepatocytes and non-parenchymal cells.

Scientific Coordinator: Robert Kavlock (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/virtual_liver/

Other components of the EPA's Computational Toxicology Research Program

Besides the above-mentioned projects that operate in the related fields of the **SEURAT-1** Research Initiative, the Computational Toxicology Research Program comprise further components that will be just briefly mentioned:

The project "Determining Uncertainty" develops and uses advanced statistical tools to analyse the uncertainty of model predictions in biological models.

The ExpoCast™ project has its focus on the environmental fate of chemicals to assess exposure routes. The project is closely related to ToxCast with the common goal to establish a list of priority chemicals to be further tested and/or regulated.

The ToxPi project provides a platform to interconnect the information about toxicity pathways, dose estimates and chemical structures from other projects of the programme.

The v-Embryo project has its focus on developmental toxicity with the overall goal to develop

prediction techniques for improved understanding of how environmental influences may impact unborn children. The project interacts with the ToxCast and the v-Liver projects.

Finally, STAR is a programme that funds large research centres in specific areas of national concern including health effects, but also global change and ecosystem assessment and restoration.

Scientific Coordinator: Robert Kavlock (Director of EPA's National Center for Computational Toxicology, Research Triangle Park, USA)

More information: http://www.epa.gov/ncct/research_projects.html

PSTC: Predictive Safety Testing Consortium (InnoMed)

The PSTC is a public-private partnership supervised by the Critical Path Institute (C-Path) as an independent, non-profit institute, which was created by the University of Arizona and the U.S. Food and Drug Administration (FDA) in 2005. PSTC provides a platform for pharmaceutical companies to share and validate each other's safety testing methods under advisement of the United States Food and Drug Administration (FDA) and its European counterpart, the European Medicines Agency (EMA). PSTC is creating new tools for safety assessment in drug development useful for pharmaceutical scientists, regulators, and clinicians. Currently, the PSTC has 17 corporate members.

The PSTC has two main objectives: (1) The identification and cross-qualification of new and improved pre-clinical safety testing methods through a collaboration of scientists from the pharmaceutical industry, academia, and regulators (FDA, EMA, Japanese Pharmaceuticals and Medical Devices Agency). (2) The facilitation of the development of new regulatory processes for approving such testing methods. The PSTC is subdivided into six working groups addressing the following areas of safety: Kidney injury, liver injury, skeletal muscle injury, vascular injury, cardiac hypertrophy, and carcinogenicity.

Director: Elizabeth Walker (Critical Path Institute, Tucson, USA)

More information: http://www.c-path.org/pstc.cfm

HESI: Health and Environmental Sciences Institute

The Health and Environmental Sciences Institute (HESI) is a non-profit, scientific organisation located in Washington D.C. HESI was established in 1989 as a global branch of the International Life Science Institute (ILSI). HESI's intention is to bring together different research groups from industry, government, and academia to advance the understanding of scientific issues in the field of human health, toxicology, risk assessment and the environment. HESI developed

a programme for laboratory studies, workshops and conferences that is currently used by 43 companies representing different branches of the chemical industry. This programme is co-financed through grants from the private sector as well as from various international regulatory institutions.

Executive Director: Michael P. Holsapple (Health and Environmental Science Institute, Washington D.C., USA)

More information: http://www.hesiglobal.org/

CAAT: Centre for Alternatives to Animal Testing

The Centre for Alternatives to Animal Testing (CAAT) is located within the Johns Hopkins Bloomberg School of Public Health in Baltimore. It was established in 1981 through a grant from the Cosmetic, Toiletry, and Fragrance Association (CTFA). Similarly to the European counterpart described above, CAAT's vision is to be a leading force in the development and use of methods following the 3R's principle (reduction, refinement and replacement) in all involved sectors (research, testing and education). Consequently, CAAT supports research for the development and validation of new *in vitro* test methods and other alternatives, organises discussion to enhance acceptance of such new methods, distributes information to academia, government, industry and the general public (for instance through the ALTEX journal), and organises training courses in the application of innovative methods in toxicity testing.

More information: http://www. caat.jhsph.edu

JAPAN

JaCVAM: Japanese Center for the Validation of Alternative Methods

JaCVAM is part of the Office for New Testing Method Assessment in the Division of Pharmacology of the Japanese National Biological Safety Research Centre (NBSRC) and the National Institute of Health Sciences (NIHS). JaCVAM is responsible for the evaluation of innovative testing methods following the 3Rs principle in the field of chemical toxicity screening and thereby for chemical safety assessment in Japan. JaCVAMS agenda comprises also the establishment of guidelines for alternative testing methods, with special emphasis on international collaborations for the development of harmonised experimental protocols (e.g., correlation with OECD guidelines). For that, JaCVAM organises international workshops and disseminates the respective information regarding alternative testing methods. Furthermore, representatives of the US National Toxicology Program, Health Canada, Japan (JaCVAM) and the EU (ECVAM) signed a memorandum of cooperation in 2009 with the aim to establish an International Cooperation on Alternative Test Methods (ICATM). This was done in order

"to expand and strengthen cooperation, collaboration and communication among national validation organisations on the scientific validation and evaluation of new alternative testing methods proposed for regulatory health and safety assessments" (Memorandum of Cooperation, http://jacvam.jp/en_effort/en_icatm.html).

More information: http://jacvam.jp

Percellome Project

The Percellome Project was funded by the National Institute of Health Sciences (NIHS) as part of the Toxicogenomics Projects. It has generated an extended database and information system for the mechanism-based prediction of toxic effects of chemicals. All in all, more than 90 different chemicals were studied based on microarray studies. The microarrays were developed in order to analyse molecular reactions upon exposure to chemicals, for instance alterations in gene expression pattern. Initially, Percellome was designed with the aim to compare data of the transcriptom from different studies covering a variety of *in vitro* methods. The direct data comparison was anticipated through a normalization of mRNA expression values. Based on that, initial molecular events triggering adverse effects were elucidated potentially allowing the faster and more effective development of *in vitro* testing methods.

More information: http://www.nihs.go.jp/tox/TTG_Archive.htm

5.3.3 Meetings and Symposia

Focus on Alternative Testing

1st International Forum on Cosmetic Technology and Applications – Alternatives to Animal Experimentation for Cosmetics

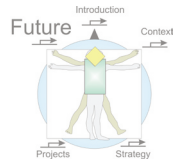
Date: 11 – 12 April 2011

Location: Beijing, China

The forum brought together representatives from relevant companies and guilds, researchers and experts from research institutes in the European Union, America, Japan and South Korea, as well as Chinese experts from cosmetic industry and government officials from relevant Chinese supervision departments, to exchange ideas and conduct discussions on alternatives to animal experimentation for cosmetics.

Main themes for discussions were:

- The background of alternatives to animal experimentation for cosmetics.
- Current application situation of alternatives to animal experimentation for



cosmetics in different countries.

➠ Communications on alternatives to animal experimentation for cosmetics, including eye irritation, cosmetics toxicology, *in vitro* organ models, test for cell function and cytotoxicity *in vitro*, etc.

➠ Prospect of alternatives to animal experimentation for cosmetics.

In Vitro Testing Industrial Platform (IVTIP), Spring Meeting 2011

Date: 26 – 28 April 2011

Location: Monaco

IVTIP is an informal forum of companies with a genuine and active interest in *in vitro* testing methods to be used in regulatory/safety testing, as well as for early decision-making in compound discovery and development. IVTP's members represent companies in the following sectors: chemicals, cosmetics, consumer products and pharmaceuticals. IVTIP is also supportive of applying opportunities resulting in the reduction of the number of animals and/or allowing for refinement of existing animal experiments. Besides the 3Rs, IVTIP actively endorses a 4th R: responsibility towards animals as well as towards the growing demand by society for better ways of assuring safety. The theme of the Spring Meeting 2011 was "*In vitro* reconstructed human tissue models as alternatives to animal testing: applicability and limitations".

More information: http://www.ivtip.org/

DNT3 Developmental Neurotoxicity. 3rd International Conference on Alternatives for Developmental Neurotoxicity Testing

Date: 10 – 13 May 2011

Location: Varese, Italy

Developmental Neurotoxicity is an issue of growing concern in the context of chemical exposure. The developing human nervous system is susceptible to toxicants, and exposure during development may cause lasting neurological deficits. This conference brought together diverse stakeholders from around the globe (research scientists, regulators, industry representatives, academics, paediatricians etc). The theme of the conference was "Advancing the science of developmental neurotoxicity testing for better safety evaluation".

More information: http://ihcp.jrc.ec.europa.eu/events_workshops/dnt3conference

AXLR8: Workshop 2011

Date: 22 – 25 May 2011

Location: Berlin, Germany

AXLR8 is a coordination action funded by the European Commission Directorate General for Research & Innovation (Health Directorate; Advanced Therapies and Systems Medicine Unit) under the 7th European RTD Framework Programme Health Theme. AXLR8 will provide tools and opportunities for increased networking, information exchange, problem solving, strategic planning and collaboration among a variety of scientific disciplines and stakeholder groups with the goal to accelerate the transition to a toxicity pathway-based paradigm for chemical safety assessment. AXLR8 will act as a focal point for coordination among 3Rs research projects in Europe as well as internationally.

Members of COACH from the **SEURAT-1** Research Initiative participated in the workshop and presented the long-term research strategy, which is given in Chapter 3 of this Annual Report. It is expected that the AXLR8 workshop will become a very important platform for fostering international collaborations between the **SEURAT-1** Research Initiative and the related international activities.

More information: http://axlr8.eu/

8th World Congress on Alternatives and Animal Use in Life Sciences

Date: 21 – 25 August 2011

Location: Montreal, Canada

The Congress provides a forum supporting the ethical use of animals in chemical testing, as well as scientific exchange regarding the development of innovative experimental methods. The Canadian tradition of consensus-building inspired the Congress motto 2011: “The Three Rs – Together it’s possible”.

Main themes for discussions are as follow:

- Safety and Efficacy Testing of Chemicals, Pharmaceuticals and Biologicals
- Policy/Law on Animal Use, Public Engagement and Ethics Review
- Incorporation of the Three Rs in Education and Training
- Animal Welfare for Refinement and High Quality Science
- Replacement and Reduction in Basic Research

More information: http://www.wc8.ccac.ca/pages/welcome

OTHERS IN THE FIELD

ECHA`s 6th Stakeholders` Day

Date: 18 May 2011

Location: Helsinki, Finland

The European Chemicals Agency (ECHA) organised as its biggest external event its 6th Stakeholders` Day to inform participants involved in REACH (Registration, Evaluation, Authorisation of Chemicals) and CLP (Regulation on Classification, Labelling and Packaging of Substances and Mixtures) about ECHA`s activities and new developments. Providing member states and other European institutions with scientific advice concerning the safety and the socio-economic areas in view of the use of chemical substances, ECHA`s 6th Stakeholders` Day also enabled the exchange of new ideas and expectations in the field of REACH and CLP processes.

Main themes for discussions were:

- Registration, Authorisation and Restriction
- Downstream user obligations
- Evaluation and Dissemination
- One-to-one discussion between participants and ECHA staff about REACH- and CLP-related issues

More information: http://echa.europa.eu/news/events/6th_stakeholders_day_en.asp

47th Congress of the European Societies of Toxicology

Date: 28 – 31 August 2011

Location: Paris, France

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). The theme of the 2011 congress is “Safety Evaluation: A Translational Science”.

Main themes for discussions are:

- Regulatory toxicology and risk assessment
- Clinical toxicology
- Safety of food, drugs, cosmetics, biocides, pesticides, metals

- Environmental and occupational toxicology
- *In vitro* toxicology
- Nanomaterials
- Immunotoxicology
- OMICS
- Genotoxicity and carcinogenesis
- Organ toxicities
- Mechanisms of toxicity

More information: http://www.eurotox2011.com/

EPAA and ecopa

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).

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, such as the "28th Workshop of SCCT and the FINCOPA seminar: "Towards Toxicity Testing Without Animals" that is going to be held on 21 – 23 September 2011 in Tampere, Finland.

More information: http://ec.europa.eu/enterprise/epaa/index_en.htm

http://www.ecopa.eu/

5.4 *Consilio et animis:* Inducing and Accelerating Co-operations between European-based Public-Private Research Partnerships in Toxicological Risk Assessment

Ian Cotgreave

Assessing risks to human health from chemical exposures, be them in the form of cosmetic ingredients, pharmaceutical preparations or chemicals in the ambient and work environments, lies centrally at the cross roads between economic development, improving the quality of life and conserving the natural environment. Much of this risk assessment has hitherto been performed using experimental animal bioassays and pathological examination to determine the relevant dose-response relationships in the toxicological profile of a particular compound, and assess margins to potential human exposure. However, we are today standing on the verge of paradigm shift in risk assessment practices, whereby a shift towards toxicity pathway-based assessments from non-animal based testing may provide much of the information required for formulating informed risk decisions. These opportunities are being provided by a number of separate advances in areas of science ranging from the development of computational tools to harnessing the pluripotency potential of human stem cell biology.

Harnessing the full potential in this paradigm shift will require directed coordination of research resources at a number of levels within society. These coordinative efforts are well illustrated in the more traditional formation of academic research consortia, as is common within the various European Framework programmes, as well as in consortia formed between academic and industrial partners, as exemplified by the Innovative Medicines Initiative. However, due to the diversity of interested partners in the area of chemical risk assessment, there is a risk that research resources emanating from independent funding bodies are spent on similar or even over-lapping aspects of scientific development. Indeed, within the European Union, this is occurring as we speak. For instance, at the same time the SEURAT programme, supported by the European Commission and the Cosmetics Industry Trade Association Colipa, is addressing the area of predicting chronic human liver toxicity using human stem cell technology, pathway mapping technologies and advances in materials technology, the Stem Cell for Safer Medicines consortium (*SC4SM, 2011*), supported by the UK government and the pharmaceutical industry, is trying to create better prediction of acute human liver toxicity using similar tools and technologies to those in the **SEURAT-1** Research Initiative. This is further complicated by diverse efforts within the 6th (*European Commission, 2011a*) and 7th

(*European Commission, 2011b*) Framework Programmes from the European Commission, many oriented towards prediction of human risks from chemicals in the environment, and the very recent launch of a new IMI call on the use of stem cell technologies in better prediction of human safety and efficacy of emerging drug candidates (*IMI, 2011*).

Clearly there is an urgent need to not only coordinate the work performed within individual scientific consortia or clusters of consortia within a particular financial support framework, but also in supra-coordination of effort between these individual consortia and clusters, irrespective of the origin of the need for risk assessment. Effective coordination at this higher level will reside in ability to conceptualise and utilise commonality in purpose, but also in developing and leveraging more effective communication between the scientific advisory panels and funding bodies supporting these individual efforts. In making initial steps towards this, the AXLR8 co-ordination project funded under the 7th EU Framework Programme was established **(***AXLR8, 2011***)** with the goal to enhance networking and collaboration among scientists, regulators, and key stakeholders at European and international levels. The **SEURAT-1** Research Initiative and its Scientific Expert Panel stands prepared to support these efforts by using and expanding the interactive platform provided by AXLR8. This has already been established through active participation of members from the **SEURAT-1** coordination project COACH on the AXLR8 workshop held on 23-25 May 2011 in Berlin, Germany. By that, the **SEURAT-1** Research Initiative will play a major role in instigating scientific and infrastructural interactions with other European consortia, on the critical path for success in moving into the new risk assessment paradigm in the service of our society.

References:

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European Commission (2011b): Seventh Framework Programme.-
http://cordis.europa.eu/fp7/ (accessed on 1 July 2011).

IMI (2011): Innovative Medicines Initiative.-
http://www.imi.europa.eu/ (accessed on 1 July 2011).

AXLR8 (2011): Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development.-
http://axlr8.eu/ (accessed on 1 July 2011).

Glossary

3Rs
Reduction, replacement, refinement - defined by Russel & Birch 1959.

ADME
Absorption, Distribution, Metabolism, and Excretion. ADME describes the disposition of a pharmaceutical compound within an organism.

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 enrichement analysis of genomic regions to analyse the presence or relative distribution of histone-modifications and histone variants at and across genomic regions.

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).

CYP
Cytochrome-P450.

EB
Embryoid body.

EC
Endothelial cell.

ECG
Electrocardiogram.

Ecopa
European Consensus Platform for 3R Alternatives.

ENCODE
ENCyclopedia Of DNA Elements, NHGRI programme to identify all functional elements in the human genome sequence in the human genome http://genome.ucsc.edu/ENCODE/.

ECVAM
European Centre for the Validation of Alternative Methods.

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 fusiogenic protein VSVG. Vesicles (“Gesicles” where the G stands for the G viral protein) formed and released by those producing cells are, then, both much more numerous and very prone to fusion with cell membranes. Engineering producing cells with constructs encoding proteins of interest leads to packing of well translated and processed proteins in gesicles, providing a way to produce and transfer proteins into target cells where normal function has been well demonstrated.

GFP
Green fluorescent protein.

GLP
Good laboratory practice.

GMP
Good manufacturing practice.

Gold Compound
A well characterised compound for toxicity testing.

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.

HSC
Hepatic stellate cells.

HSEC
Hepatic sinusoidal endothelial cells.

HTS
High-Throughput-Screening.

***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.

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.

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.

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.

MRM
Multiple Reaction Monitoring (MRM), simultaneous quantification of a large number of peptides (several hundreds) in transcriptomics (Toxicoproteomics).

mRNA
Messenger RNA.

MS
Mass spectrometry.

M.SssI
DNA methyltransferase from Spiroplasma sp. with the DNA sequence specificity CpG.

MTT assay
Assays for measuring the activity of enzymes that reduce 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or close dyes (XTT, MTS, WSTs) to formazan dyes, giving a purple color. Used to assess the viability (cell counting) and the proliferation of cells (cell culture assays), as well as cytotoxicity.

NIH reference map
Epigenome reference map: A program launched by the NIH to uncover the epigenomic landsacape across human cells.
http://www.roadmapepigenomics.org/

NMR
Nuclear magnetic resonance.

NOAEC
No observed adverse effect concentration.

NOAEL
No observed adverse effect level.

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.

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

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.

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.

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.

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 adjacently connected atoms, in a molecule. A substructure associated with the presence of a biological activity is sometimes called a structural alert.
A SAR can also be based on the ensemble of steric and electronic features considered necessary to ensure the intermolecular interaction with a specific biological target molecule, which results in the manifestation of a specific biological effect. In this case, the SAR is sometimes called a 3D SAR or pharmacophore.

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.

SOP
Standard Operating Procedure.

Tanimoto criteria
Molecular similarity criteria for chemicals based upon Tanimoto Coefficients.

TBBB
The ToxBank BioBank (TBBB) will establish a banking information resource for access to qualified cells, cell lines (including stem cells and stem cell lines), tissues and reference materials to be used for *in vitro* predictive toxicology research and testing activities.

TBCR
The ToxBank Chemical Repository will ensure the availability of test compounds to researchers of the **SEURAT-1** Research Initiative.

TBDW
The ToxBank Data Warehouse will establish a centralised compilation of data for systemic toxicity.

TBGCD
The ToxBank Gold Compound Database will provide a information resource servicing the selection and use of test compounds.

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.

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.

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 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 the European Cosmetics Association (Colipa) 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 research projects focusing on the development of new non-animal test methods in the field of repeated dose systemic toxicity.

- This is the first 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