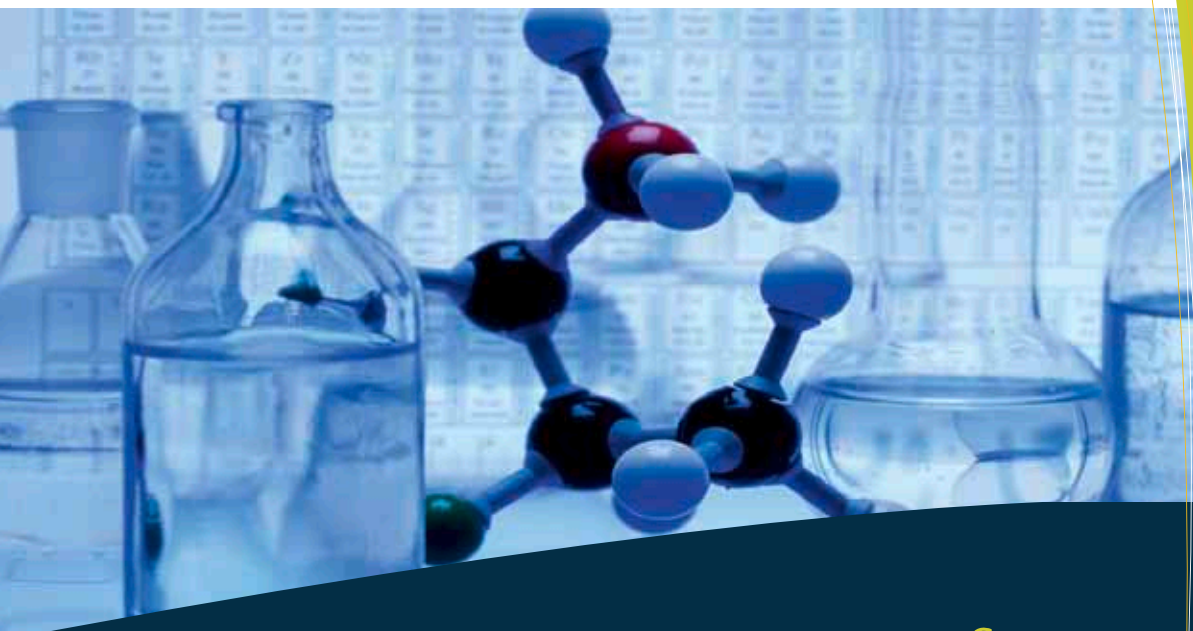# AXLR8

ALTERNATIVE TESTING STRATEGIES

PROGRESS REPORT 2011

& AXLR8-2 Workshop Report on a
'Roadmap to Innovative Toxicity Testing'

Advanced Therapies and Systems Medicine

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The AXLR8 Consortium

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& AXLR8-2 Workshop Report on a 'Roadmap to Innovative Toxicity Testing'

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EXECUTIVE SUMMARY

This publication is the fourth in a series of reports summarising the progress of research to advance the '3Rs' concept (Replacement, Reduction and Refinement of animal testing) funded by the European Commission Directorate General for Research & Innovation (DG R&I) within the Health theme of the Sixth and Seventh European Research Framework Programmes (FP6 and FP7, respectively), as well as at the international level. Prepared by the FP7 co-ordination and support project AXLR8 (pronounced 'accelerate'), this report is a follow-up to earlier Commission publications and the first AXLR8 Progress Report 2010. AXLR8 was established to set up a strong, co-ordinated research area through improvement of communication and co-operation between the scientists of existing and past FP, EU Member State and international research projects in the topic area. The need for co-ordination was expressed by the respective project co-ordinators and participating scientists.

AXLR8 has the objective to monitor the progress of EU-funded FP6/7 research projects aimed at the development of alternative testing methods, to identify gaps in knowledge, to define priority research needs, and to prepare, publish and disseminate progress reports on an annual basis. To achieve this goal, AXLR8 has appointed a Scientific Panel comprised of co-ordinators of EU FP6/7 projects on 'alternative testing strategies' and external experts from academia, regulatory authorities and industry involved in chemicals, pharmaceuticals, cosmetics and consumer products sectors. An essential element of the AXLR8 project is the organisation of annual workshops to provide a scientific platform for high-level information exchange and critical discourse among co-ordinators of EU-funded projects and independent European and international scientists on progress achieved in developing alternative testing strategies, as well as

challenges, needs, and priorities for future EU research.

AXLR8 is particularly aimed at accelerating a transition in Europe toward a 'toxicity pathway'-based paradigm[1-5] for chemical safety assessment through internationally co-ordinated research and technology development with the common goals of improved health and environmental protection, positioning the EU on the leading-edge of a rapidly developing global research area, and working toward replacement of animal testing.

A major activity of AXLR8 during the past year was organising the second annual workshop (AXLR8-2), which was held in Berlin, Germany from 22-25 May 2011 with a focus on developing a 'roadmap to innovative toxicity testing'. Among the more than 50 invited participants were representatives of projects funded by the FP6/7 health and environment programmes, the heads of Member State

centres on alternatives to animal testing, the leaders of international efforts to establish advanced molecular toxicology from the United States and Japan, and members of the AXLR8 Scientific Panel and Consortium. An executive summary of the AXLR8-2 workshop report on a 'roadmap to innovative toxicity testing' was published on the *axlr8.eu* website within one week of the workshop, and is included in the final section of this document.

Chapter 1 is the introduction to the Progress Report 2011, in which the key objectives and deliverables of the AXLR8 project are described, including major activities during the first two years.

Chapter 2 consists of annual progress reports, and in some cases final reports, prepared by the co-ordinators of FP6/7-funded projects. Because most of these projects have been described in detail in earlier reports, previously published content will not be repeated. Readers are referred to Progress Reports 2008-2010, which are available online at *axlr8.eu/publications*.

Chapter 3, the core of the 2011 Progress Report, is devoted to the AXLR8-2 workshop and 'roadmap to innovative toxicity testing'. The workshop began with a public satellite meeting, providing an overview of current EU and global research efforts on advanced molecular methods in toxicity testing and the modelling of human diseases. Thereafter, co-ordinators of EU FP6 and FP7 projects reported on progress achieved during the past year in the development of alternative

[1] US National Research Council. Toxicity Testing in the 21st Century: A Vision and Strategy. Washington, DC: National Academies Press, 2007.

[2] Berg N, De Wever B, Fuchs HW, et al. Toxicity in the 21st century – working our way towards a visionary reality, 2010, www.ivtip.org/images/IVTIP_publication-final.pdf.

[3] Ankley GT, Bennett RS, Erickson RJ, et al. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environ Toxicol Chem. 2010; 29, 730-41.

[4] US Environmental Protection Agency. NextGen, Website epa.gov/risk/nexgen, 2010.

[5] EPAA. Final Report – Workshop on Computational Chemistry and Systems Biology. Website http://ec.europa.eu/enterprise/epaa/2_activities/2_1_science/ws_comp_chem_final.pdf, 2011.

testing strategies. Plenary presentations focused on the toxicity pathway concept in general, with case studies in the areas of reproductive toxicity and skin sensitisation (allergy). Workshop participants were divided into three breakout groups for a focused discussion of the scientific state-of-the-art and of knowledge gaps and priorities for future EU research funding in order to develop a 'roadmap to innovative toxicity testing'. In the first breakout group, general building blocks for a pathway-based paradigm were discussed, while the other two groups examined reproductive toxicity and skin sensitisation as case studies. Chapter 3 also includes manuscripts from workshop presentations, summary reports from the three breakout groups, and the recommendations of the AXLR8 Scientific Panel for a 'roadmap to innovative toxicity testing'—also termed 'safety evaluation ultimately replacing animal testing' ('SEURAT-2') by DG R&I.

Thus the AXLR8 Progress Report 2011 is again serving the ultimate goal of AXLR8 to set the stage for an in-depth discussion on how to accelerate the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development.

1 INTRODUCTION

It is the aim of the AXLR8 project to lay the groundwork for a transition in toxicology toward a more pathway-based *in vitro* and computational approach through enhanced networking and collaboration among scientists, regulators and other key stakeholders at European and international levels.

To date almost € 140 million in funding has been provided under the 6th and 7th EU Framework Programmes to advance the development and validation of 3Rs methods and testing strategies for regulatory purposes (Table 1). These funding activities have been 'policy-driven' by Directive 86/609/EEC for the protection of animals used for experimental and other scientific purposes, as well as the 7th Amendment of the EU Cosmetics Directive, and the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation—all of which provide explicit legislative mandates to replace regulatory toxicity testing in animals with non-animal approaches. To achieve this goal, a variety of large-scale 'integrated projects' have been funded by DG R&I, in which scientists from academia, industry and government collaborate toward animal replacement on the level of classical toxicological endpoints such as acute and repeated dose toxicity. In 2011, in a joint venture between DG R&I and the European Cosmetics Association (COLIPA), € 50 million was raised for the 'replacement of *in vivo* repeated dose systemic toxicity testing' with the long-term target of 'safety evaluation ultimately replacing animal testing' (SEURAT).

Similar funding programmes have been launched by 3Rs centres in several EU Member States and also in Japan, the United States, and elsewhere. Monitoring the scope

Table 1. Overview of funding for 'alternative testing strategies'.

Project name	FP	Total awarded grant (€)	From	To
Predictomics	6	2,259,754	2004	2007
CONAM	6	150,000	2004	2007
ReProTect	6	9,100,000	2005	2009
Sens-it-iv	6	10,999,700	2005	2010
ACuteTox	6	9,000,000	2005	2009
EXERA	6	2,173,492	2005	2010
TOXDROP	6	1,615,888	2005	2006
carcinoGENOMICS	6	10,440,000	2006	2011
VITROCELLOMICS	6	2,940,000	2006	2009
EUPRIM-NET	6	4,800,000	2006	2010
MEMTRANS	6	1,900,000	2006	2009
INVITROHEART	6	2,701,611	2007	2009
LIINTOP	6	2,933,291	2007	2009
ARTEMIS	6	1,984,000	2007	2010
COMICS	6	2,189,385	2007	2010
SCARLET	6	112,840	2007	2008
ForInViTox	6	288,850	2007	2009
InViToPharma	6	578,000	2007	2008
OSIRIS	7	10,000,000	2007	2011
PREDICT-IV	7	11,330,907	2008	2013
ESNATS	7	11,895,577	2008	2012
NanoTEST	7	3,933,271	2008	2011
OpenTox	7	2,975,360	2008	2011
START-UP	7	317,984	2008	2010
AXLR8	7	555,223	2010	2013
CHEMSCREEN	7	3,500,000	2010	2014
SCR&Tox*	7	4,700,000	2010	2015
HeMiBio*	7	4,700,000	2010	2015
DETECTIVE*	7	4,340,000	2010	2015
COSMOS*	7	3,340,000	2010	2015
NOTOX*	7	4,850,000	2010	2015
ToxBank*	7	1,560,000	2010	2015
COACH*	7	1,500,000	2010	2015
Total funding		**136,665,133**		

* Contribution by the DG Research & Innovation, matching funds are provided by COLPIA.

and progress of these multidisciplinary initiatives and providing opportunities for communication and dissemination of research results is becoming increasingly important for identifying knowledge gaps, areas of overlap, and opportunities for synergies. The AXLR8 project is supporting this process by providing up-to-date information to DG R&I on the international scientific state-of-the art and on needs and opportunities for future funding of research in Europe.

AXLR8 Consortium

The past two decades have seen unprecedented scientific and technological advances, including the birth of functional genomics, the explosive growth of computing power and computational biology/bioinformatics, the establishment of robotic platforms for high-throughput screening of chemicals, and the sequencing of the human genome. Together these advances have triggered a revolution in molecular biology, and have made available a wide range of new tools for studying the effects of chemicals on cells, tissues and organisms in a rapid and cost-efficient manner. In the United States, this convergence of factors, coupled with increased recognition of the limitations of conventional *in vivo* tests and the need to evaluate the safety of an increasingly large number of chemical substances and mixtures, has led authorities to call for shift in toxicity testing towards the elucidation of 'toxicty pathways' at the cellular level—an approach termed '21st century toxicology'.

Recognising the growing need of a focal point for monitoring the increasing number of 3Rs and related multidisciplinary research initiatives worldwide, Humane Society International/United Kingdom (HSI), the Freie Universität Berlin, and the Centre for Advanced R&D on Alternative Methods at the Flemish Institute for Technological Research (CARDAM-VITO) formed the AXLR8 Consortium.

The core of the AXLR8 project is a series of annual workshops, which bring together the co-ordinators of EU FP6/7-funded research projects, international scientists, and regulatory and corporate end-users of test methods, to discuss the progress of ongoing activities, identify knowledge gaps and opportunities for synergies, and help to streamline priorities for future research. The AXLR8 project began its activity in 2010 and will continue through 2013.

AXLR8 Goals & Objectives

The goal of the AXLR8 project is to lay the groundwork in Europe for a transition in toxicology towards a more mechanistic, cell- and computer-based approach, and to direct Europe into a leading position in this advanced research area in the life sciences.

Realisation of this goal will depend on the project's success in fostering effective information sharing and collaboration among research initiatives both at the European and global level. To achieve this goal the following specific objectives are pursued:

- Monitoring the progress of DG R&I-funded health research projects and publication of annual progress reports.
- Establishment of a Scientific Panel with experts from the EU corporate, governmental and academic sectors, and leading international scientists, to provide an external expert perspective on future research needs and priorities.
- Organisation of annual scientific workshops to bring together the leaders of EU-funded research projects, members of the AXLR8 Scientific Panel and other selected experts to discuss the progress of currently funded projects, identify knowledge gaps, and recommend strategic priorities for future research in Europe.
- Promotion of scientific, stakeholder and public awareness and communication on the outcome of AXLR8 workshops and on '21st century' approaches to toxicology and risk assessment.
- Promotion of active engagement by regulators to ensure that authorities are kept informed about progress at the research and development level and that regulatory considerations are understood and fed back to the scientists developing 3Rs methods in order to encourage and lay the groundwork for more efficient acceptance of alternative approaches in the future.

AXLR8 Scientific Panel

A cornerstone of the AXLR8 project is its Scientific Panel, which is a platform for information exchange and critical discourse with co-ordinators of EU DG R&I-funded FP6/7 health projects and independent experts in toxicology (*in vitro*, *in silico* and mammalian), systems biology and bioinformatics and risk assessment. The AXLR8 Scientific Panel meets at the annual AXLR8 workshops and supports the AXLR8 Consortium in monitoring research progress and in the identification of future needs and priorities. The current membership of the AXLR8 Scientific Panel is listed in Table 2.

AXLR8 Workshops

The first AXLR8 workshop (AXLR8-1) was held in Potsdam, Germany, from 31 May to 2 June 2010. Participation was limited to the co-ordinators of FP6/7-funded health projects, representatives of national 3Rs centres in EU Member States and Japan, leading US scientists working to advance the 21st century toxicology approach, representatives from the European Commission DGs R&I and Joint Research Centre, and members of the AXLR8 Scientific Panel and the Consortium.

The second AXLR8 workshop (AXLR8-2) was held in Berlin, Germany from 22-25 May 2011 with a focus on developing a 'roadmap to innovative toxicity testing'. Among the more than 50 invited participants were representatives of projects funded by the FP6/7 health and environment programmes, the heads of

Table 2. Members of the AXLR8 Scientific Panel.

European Experts

Name	Institution	Sector	Country
Nathalie Alépée	L'Oréal	Cosmetics	FR
Patric Amcoff	OECD	Intergovernmental	SE
Jürgen Borlak	Fraunhofer Institute	Research institute	DE
Steffen Ernst		Pharmaceuticals	SE
Julia Fentem	Unilever	Consumer products	UK
Ellen Fritsche	University of Duesseldorf	Academic	DE
Joanna Jaworska	Procter & Gamble	Consumer products	BE
Robert Landsiedel	BASF	Chemicals	DE
Maurice Whelan	European Commission	Governmental	IT

International Experts

Name	Institution	Sector	Country
Harvey Clewell	The Hamner Institutes for Health Sciences	Research institute	US
Robert Kavlock	Environmental Protection Agency	Governmental	US
Hajime Kojima	National Institute of Health Sciences	Governmental	JP

Representatives of EU-Funded Projects

Name	Institution	Project	Country
Manuel Carrondo	Instituto de Biologia Experimental e Tecnologica	VITROCELLOMICS	PT
Barry Hardy	Douglas Connect	OpenTox, ToxBank	CH
Jürgen Hescheler	Universität Köln	ESNATS, DETECTIVE	DE
Jos Kleinjans	Maastricht University	carcinoGENOMICS, DETECTIVE	NL
Carl-Fredrik Mandenius	Linköping University	INVITROHEART, VITROCELLOMICS	SE
Michael Schwarz	Universität Tübingen	ReProTect, COACH	DE
Flavia Zucco	Consiglio Nazionale delle Richerche	LIINTOP	IT

Member State centres on alternatives to animal testing, the leaders of international efforts to establish advanced molecular toxicology from the United States and Japan, and members of the AXLR8 Scientific Panel and Consortium. Based on a focused discussion of the scientific state-of-the-art and of knowledge gaps and priorities for future EU research funding, the participants developed a 'roadmap to innovative toxicity testing'. To achieve this goal, building blocks for a pathway-based paradigm were discussed and two case studies focused on reproductive toxicology and skin sensitisation. The discussion and recommendations of the AXLR8 Scientific Panel and the AXLR8 Consortium for a 'roadmap to innovative toxicity testing' are published in the final section of this document. They may serve to initiate the discussion on future funding of a 'SEURAT-2 ('safety evaluation ultimately replacing animal testing') project by DG R&I.

AXLR8 Outreach & Dissemination

A substantial component of the AXLR8 work programme is dedicated to outreach and engagement of a wider audience of stakeholders. This is because the ultimate success of this co-ordination initiative will depend heavily on securing acceptance by a variety of key stakeholders, in particular scientists and regulators, but also policy-makers, public interest NGOs, and the European public at large.

Scientific workshops and other forums in which AXLR8 has been an invited contributor include the following:

Figure 1. Mainstream media coverage of AXLR8 at guardian.co.uk/science/2010/dec/31/animal-research-alternatives

- AXLR8 Workshops 2010-11 and Info Forum
- 8th World Congress on Alternatives and Animal Use in the Life Sciences
- Competent Authorities for REACH and Classification and Labelling
- ecopa Workshops 2010-11
- ESTIV-EUSAAT Congress 2010
- ESTIV 2011
- FELASA-SCAND 2010 Symposium
- Human Toxicology Project Symposium 2010
- ILSI-Europe TTC Workshop
- i-SUP 2010 Conference
- JSAAE-JaCVAM Workshop 2011
- Meeting with the German Ministry for Research and Education
- OECD Advisory Group on Molecular Screening and Toxicogenomics

- OECD Joint Meeting Special Session on Animal Welfare
- OECD Working Party on Manufactured Nanomaterials Steering Group on Alternatives (SG7)
- SEURAT-1 Kick-Off Meeting 2011

AXLR8's activities have also been reported on in the mainstream media, including *The Guardian* (Figure 1) and *The Telegraph*, various trade journals and radio programmes, and in the newsletters of the European Society for Toxicology *In Vitro*, ecopa, and AltTox.

In addition, the website *axlr8.eu* provides 'one stop shopping' for links to 3Rs-relevant EU research projects and annual progress reports, key international initiatives and publications, and a listing of upcoming meetings and conferences. There you can also register for the periodic AXLR8 e-newsletter, which is sent to a distribution list of over 1,400 subscribers.
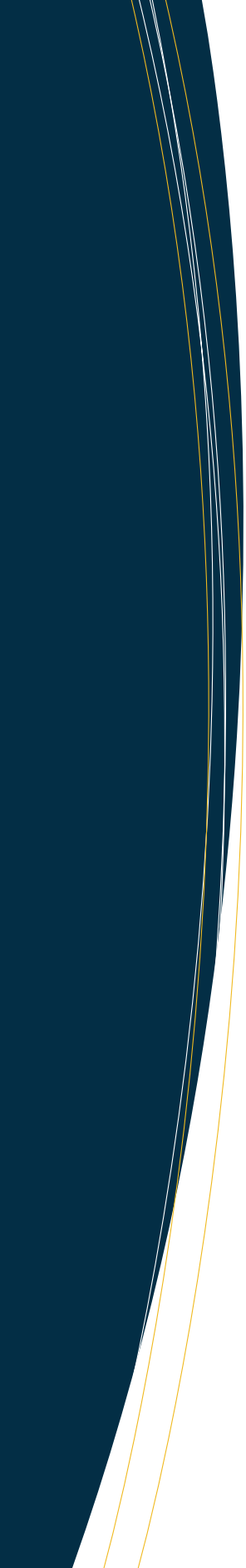
2 PROGRESS REPORTS FROM EU-FUNDED PROJECTS

Projects supported in 2010 by the EU FP6/7 health programme

and other funding streams within the scope of 'alternative testing strategies' cover a wide spectrum of advanced innovative methods. These include:

1. Cell-based technologies
2. Integrated testing strategies
3. 'Omics, bioinformatics and computational biology
4. Computational modelling and estimation techniques
5. High-throughput techniques

The length of the individual project reports is quite variable for two reasons: Several FP6 projects ended during the period of reporting, while most of the FP7 projects have only recently begun. Therefore, more space has been reserved in the 2011 Progress Report for the final reports of FP6 projects. In addition, the huge multi-centre 'Integrated Projects' (IPs) with up to 30 participating institutions also require more extensive coverage than the smaller 'Specific Targeted Research Projects' and 'SME-Specific Targeted Research Projects'.

In this chapter the FP6 and FP7 projects are presented alphabetically.

ACuteTox

Optimisation & Pre-Validation of an *In Vitro* Test Strategy for Predicting Human Acute Toxicity

Contract number: LSHB-CT-2004-512051
Project type: Integrated Project (FP6)
EC contribution: € 9,000,000
Starting date: 1 January 2005
Duration: 66 months
Website: acutetox.eu

Background & Objectives

The ACuteTox project represents the first attempt to create an integrated testing strategy based solely on *in vitro* and *in silico* methods, with the purpose of replacing animal testing for predicting human acute oral systemic toxicity and classification of chemicals into the different EU Classification, Labelling and Packaging (CLP)[1] and GHS[2] toxicity classes. At present a large number of *in vitro* models for acute toxicity testing are available. Studies, such as the Register of Cytotoxicity and the NICEATM/ECVAM Validation Study of *In Vitro* Cytotoxicity Test Methods, have shown good correlation between *in vitro* basal cytotoxicity data and rodent LD_{50} values. In addition, the MEIC (Multicentre Evaluation of *In Vitro* Cytotoxicity) programme showed a good correlation (around 70%) between *in vitro* basal cytotoxicity data and human lethal blood concentrations. This means, however, that when using the existing *in vitro* tests, a certain number of misclassifications will occur. ACuteTox aimed to identify factors that could optimise the *in vitro-in vivo* correlation for acute systemic toxicity.

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[1] For acute oral toxicity the EU CLP system toxicity categories are assigned based on the LD_{50} values: Category 1 (LD_{50} < 5 mg/kg bw, fatal if swallowed); Category 2 (LD_{50} > 5 mg/kg < 50 mg/kg bw, fatal if swallowed); Category 3 (LD_{50} > 50 mg/kg < 300 mg/kg bw, toxic if swallowed); Category 4 (LD_{50} > 300 mg/kg < 2000 mg/kg bw, harmful if swallowed); or LD_{50} > 2000 mg/kg, not classified)

[2] The United Nations Globally Harmonised System of Classification and Labelling (GHS) is an internationally standardised system for classifying chemicals. For acute oral toxicity, the GHS categories are assigned based on the LD_{50} values: GHS 1 (LD_{50} < 5 mg/kg bw, fatal if swallowed); GHS 2 (LD_{50} > 5 mg/kg < 50 mg/kg bw, fatal if swallowed); GHS 3 (LD_{50} > 50 mg/kg < 300 mg/kg bw, toxic if swallowed); GHS 4 (LD_{50} > 300 mg/kg < 2000 mg/kg bw, harmful if swallowed); GHS 5 (LD_{50} > 2000 mg/kg bw, may be harmful if swallowed; or LD_{50} > 5000 mg/kg, not classified).

The main objectives of the project included the compilation, evaluation and generation of high-quality *in vitro* and *in vivo* data on a set of reference chemicals for comparative analyses, and the identification of factors that influence the correlation between *in vitro* (concentration) and *in vivo* (dose) toxicity, particularly taking into consideration biokinetics, metabolism and organ toxicity (liver, central nervous system, kidney and blood). Moreover, innovative tools (e.g., cytomics) and new cellular systems for anticipating animal and human toxicity were explored. Ultimately, the goal was to design a simple, robust and reliable *in vitro* test strategy amendable for robotic testing, associated with the prediction models for acute oral toxicity.

General Overview of the ACuteTox Work Packages

WP1: Generation of an In Vivo Database

Principal responsibilities of WP1 were to select project reference chemicals and to generate an *in vivo* acute toxicity database for the selected reference chemicals by searching and compiling information from documented sources. WP1 was also responsible for statistical review of the compiled animal data, conceived to provide explicit insight of the LD_{50} values and establish an objective basis for projected correlation of the *in vivo* data with results from corresponding *in vitro* assays. The review made statistical analyses of variability, inter-species correlation, relevance, and predictive capacity for

toxicity classification. Results of these analyses provided a basis for performance assessment of *in vitro* methods and were relevant to the development of strategies for evaluation of chemical toxicity.

WP2: Generation of an In Vitro Database

The aim of WP2 was to review the cytotoxicity data of MEIC/EDIT and NICETAM/ECVAM employing the defined protocol approach with standardised analysis and reporting used by the NICETAM/ECVAM study. This generated a new database by extending the two studies and performing testing of additional compounds that exhibited tissue-specific toxicity.

The final outcome of WP2 is a database containing high-quality *in vitro* toxicity data obtained with defined Standard Operating Procedures (SOPs) for the assay and culture methods for the testing of up to 97 different chemicals, which were used for identification of outliers in comparison with published *in vivo* data, as well as when compared with *in vitro* data from the tissue specific test.

WP3: Database, Statistical Analysis, Assay Automation

WP3 had a central role in the project and it highly relied on input/data coming from other WPs, mainly WPs 1-2 and WPs 4-7. The main objectives of this WP were to develop of an internet-based database for storing and management of all project data; to adapt promising *in vitro* methods

to robotic screening platforms; and finally, to perform statistical analyses of *in vitro* and *in vivo* data with the final goal of identifying outliers and designing a preliminary algorithm/prediction model for prediction of acute oral toxicity.

WP4: New Cell Systems & New Endpoints

The role of WP4 in the project was to provide an innovative, alternative way to improve the predictivity of cell-based cytotoxicity assays by incorporating assays for immunotoxicity, haematotoxicity and new endpoints for cytotoxicity. Cytotoxicity assays were approached by novel methodologies based on single-cell analysis, the so-called cytomic techniques (flow cytometry and high-content assays by bioimaging) that expanded the classical cytotoxicity endpoints by introducing novel early markers of cell stress and damage. In addition, WP4 was in charge of selecting reference chemicals with immunotoxic and haematotoxic potential and participated actively to define SOPs for all methods that should be considered at the selection of the best performing method.

WP5: Alerts & Correctors in Toxicity Screening (I): Role of ADE

The overall objectives of WP5 were focused on the factors that are influencing the relationship between *in vitro* cytotoxicity data and *in vivo* doses. By assessing this relationship, the estimation of acutely toxic doses on the basis of *in vitro* toxicity has been improved. The most crucial parts of the kinetic behaviour have been studied:

absorption of compounds, distribution between blood and tissues, and the passage of special barriers. In the context of acute toxicity, the blood-brain barrier (BBB) is the most relevant special barrier and has received extra attention. Both *in vitro* and *in silico* models have been used to determine oral absorption and passage over the BBB. A crucial parameter is the use of the proper dose metric in *in vitro* experiments, i.e., the free concentration of the compound, which has also been measured. The data obtained from WP5 were used for biokinetic modelling in order to transfer the *in vitro* EC_{50} values to oral dose.

WP6 & WP7.3: Alerts & Correctors in Toxicity Screening (II and V): Role of Metabolism & Hepatotoxicity

Metabolism can result in a bioactivation phenomenon rather than in a detoxification process leading to metabolism-dependent toxicity. The main objective of WP6 was to set up of an assay to generate an 'alert' about metabolism-dependent toxicity of a given compound, by testing its effects in a metabolically competent model (primary hepatocytes) and in non-metabolising cells (cell lines) as part of a general acute cytotoxicity testing. Another important aim was to develop new software not only to determine the IC_{50}s, but to compare up to 3 dose-response curves in testing of metabolism-dependent toxicity and hepatotoxicity, and to manage and integrate all the data. Strategies based on engineered cells including expression vectors for transient and controllable expression of biotransformation enzymes

were also evaluated as a way to overcome their intrinsic limitations by generation of metabolically competent cell lines. Furthermore, *in vitro* models were evaluated regarding their metabolic capacity and the possible use of these models for generating data on liver clearance for PBBK-TD modelling (in collaboration with WP5). In addition, metabolite formation of selected compounds *in vitro* was analysed and compared to *in vivo* literature data as well as to predictions made with the METEOR software.

The ultimate goal of WP7.3 was to provide quantifiable information that could be integrated in a wider assessment of *in vitro* cytotoxicity that could anticipate *in vivo* toxicity of chemicals. Metabolically competent cells (rat hepatocytes), non-competent hepatic cells (HepG2), and non-hepatic cells (3T3 mouse fibroblasts) were used to investigate the effects of a selected list of reference compounds. In WP7.3, cell systems suitable for hepatic transport assays of anions bile acids and/or xenobiotics, including double-transfected cells and ATP dependent transport systems were also generated. Furthermore, pilot experiments using the newly developed fluorescent bile acid derivatives and cell systems were performed to determine their robustness and suitability medium-throughput testing.

WP7.1: Alerts & Correctors in Toxicity Screening (III): Neurotoxicity

Acute toxicity may be a result of impaired neuronal function, either in the peripheral or the central nervous system. The overall objective for WP7.1 was to carry out testing of the ACuteTox reference chemicals in an optimised neurotoxicity test battery, according to well defined SOPs, and to deliver high-quality *in vitro* data. The selected assays were identified as the best performing assays out of a larger set of assays in which 16 general and 10 neurotoxic reference compounds were tested. The criteria for selection were the ability of the neurotoxicity assays to (i) identify 'neurotoxic alerts', i.e., indicating alteration of neuronal function at lower concentrations than the general cytotoxicity indicated in the 3T3/NRU assay (see WP2), and (ii) 'correct' underestimated toxicity as determined by the 3T3/NRU assay. Eight different cell models for the nervous system were used for the studies on approximately 70 endpoints; in pure enzymes, native or differentiated human neuroblastoma SH-SY5Y cells, primary cultures of mouse or rat cortical or cerebellar granule neurons, mouse brain slices and mature re-aggregated rat brain cells. Several of the endpoints were analysed in more than one cell model.

WP7.2: Alerts & Correctors in Toxicity Screening (IV): Nephrotoxicity

The kidney is especially susceptible to toxicity because of its role in excreting compounds, which involves a high blood supply, concentrating, metabolising and transporting compounds. The focus in WP7.2 was on developing *in vitro* assays that reflect the role of the kidneys *in vivo*

based on functional parameters including transport and barrier function involving a transepithelial cell layer and transport.

For the measurement of nephrotoxicity, transepithelial resistance (TER) was chosen as the functional assay and the LLC-PK1 proximal tubular cell line as the test system. The functional assay reflects the *in vivo* transporting capabilities of the renal proximal tubules. The functional assay was compared to a viability assay namely the resazurin ('Alamar blue') assay under exactly the same experimental conditions.

WP8 & WP9: Optimisation of the Testing Strategy & Pre-Validation

Currently, acute oral toxicity is assessed in rats in accordance with OECD Test Guidelines 420, 423 or 425. One of the main goals of the ACuteTox project was to develop and optimise an *in vitro* testing strategy for predicting human acute oral toxicity and further pre-validate it.

The first step consisted in the identification of the methods as promising building blocks for the testing strategy on the basis of an in-depth statistical analysis of the large dataset generated with the training set of 57 compounds used during the first phase of the project.

During the pre-validation phase, the selected test methods were challenged with a new set of 32 compounds. The work performed during this challenging exercise was focused mainly on the assessment of the predictive capacity of the proposed tiered testing strategies and the identification of the combination that gives the best prediction.

Achievements

Generation of an In Vivo Database

Selection of reference chemicals

The project management opted for 97 chemicals to be selected as test items, based on statistical estimation of worthy sample size and feasibility of *in vitro* experimental testing according to available resources and projected schedule. A principal selection criterion, limiting the scope for eligibility, was availability of documented cases of acute poisoning in humans (accidental ingestion, suicidal overdose, etc.), including clinical/forensic blood concentration measurements from patients/victims. The chemical selection also incorporated nominations from project partners according to expediency of respective organ specificity research interests, including biokinetic modelling. The 97 reference chemicals cover complementary representation of GHS toxicity categories and include different generic use classes (Hoffmann et al., 2010). The chemicals were readily available from regular laboratory suppliers, facilitating direct purchase of test items by project partners.

Compilation of animal and human data

In vivo animal acute toxicity data relevant to the 97 reference chemicals were derived from published literature. Over 2,200

LD_{50} values were found, from studies of rodents (rat, mouse) and other mammals (e.g., guinea pig) including various administration routes (oral, intravenous, etc.). As available from individual studies, key attributes were extracted (i.e., species, strain and sex of animal, duration of exposure, route of administration, dose, volume applied) supplemented with clinical and necropsy reports as synoptic text.

An approach to estimate human lethal concentration (LC_{50}) values derived from time related human sub-lethal (LC_0) and lethal (LC_{100}) data determined from human acute poisoning cases was developed. Using this approach the LC_{50} values were calculated for 78 out of the 97 chemicals.

Six basal cytotoxicity assays

General cytotoxicity data were generated in mouse 3T3 Neutral Red Uptake (NRU) assay, the Normal Human Keratinocyte (NHK) NRU assay, the lymphocyte HL60 ATP assay, the liver-derived Fa32 cells with an NRU and a fluorescent total protein endpoint assay, and the liver-derived HepG2 fluorescent total protein assay. WP2 also defined solubility protocols, which have been recommended for use in all other WPs. All the data were recorded into standard Excel templates to ensure consistent calculation of the results between partners.

Once the testing was completed, the data for the IC_{50} mean values were employed in a comparison with the *in vivo* data. This analysis was conducted independently

initially by the Istituto Superiore di Sanità in Italy. All 6 basal cytotoxicity assays give similar results, which confirm the results from the MEIC study[3,4].

The AcutoxBase

The *in vivo*, *in vitro* and *in silico* data collected within the project were deposited in the AcutoxBase, which is a unique database that combines all data on acute toxicity and biokinetics of 97 selected reference chemicals. It functions as a central element of the project with regard to reporting and management of the data, and allows easy, quick and reliable exchange of the generated datasets, while enabling proper documentation and traceability of all the experimental procedures, protocols, raw data and final results. The database is provided as an internet application, thus ensuring easy access for all the ACuteTox partners all over Europe.

Identification of outliers from the in vivo-basal cytotoxicity correlations

In vitro – in vivo modelling of LC_{50} values for humans and LD_{50} values for rat were performed using different combinations of the *in vitro* cytotoxicity tests in partial

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3 Clemedson C, McFarlane-Abdulla E, Andersson M, et al. MEIC evaluation of acute systemic toxicity. Part II. In vitro results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. Altern Lab Anim. 1996(b); 24, 273-311.
4 Clemedson C, Barile FA, Ekwall B, et al. MEIC evaluation of acute systemic toxicity: Part III. In vitro results from 16 additional methods used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. Alern Lab Anim. 1998; 26, 91-129.

Table 1. Outliers identified by comparing mean IC_{50} values of 3T3/NRU cytotoxicity assay with animal LD_{50} and human LC_{50} values.

Chemical Name	Outlier in Linear Regression Between IC_{50} & rat LD_{50}	Outlier in Linear Regression Between IC_{50} & Human LC_{50}
(-) epinephrine	X	No human data
2,4-dichlorophenoxyacetic acid		X
5-fluorouracil		X
Acetaminophen		X
Atropine sulfate monohydrate		X
Cis-diammineplatinum (II) dichloride		X
Codeine		X
Cyclosporine A		X
D-amphetamine sulfate	X	No human data
Digoxin	X	X
Diqaut dibromide		X
Formaldehyde	X	X
Lindane		X
Malathion		X
Methadone hydrochloride		X
Nicotine	X	X
Ochratoxin A	X	No human data
Parathion	X	No human data
Pentachlorophenol		X
Phenobarbital	X	
Physostigmine	X	No human data
Potassium cyanide	X	
Sodium chloride		X
Sodium selenate	X	
Strychnine	X	X
Thallium sulfate	X	
Warfarin	X	

least square (PLS) multivariate regression analyses. Ultimately, the models based on the IC_{50} values from the 3T3/NRU cytotoxicity assay and rat LD_{50} or human LC_{50} values were used to identify outliers (Table 1), detected by normal probability plots. The outliers identified were defined as 0.75 log deviation of IC_{50} from LC_{50} or LD_{50}.

Linear regression analysis between in vitro IC_{50} 3T3/NRU cytotoxicity values and human LC_{50} gave an explained variance $R^2 = 0.56$ for the 67 reference chemicals, for which both sets of data were available. This R^2 value shows that additional organ-specific and biokinetic tests are needed in order to improve the predictability. Fifty-seven reference chemicals, including the identified outliers and a balanced number of non-outliers from the reference set, were then tested in more than 70 tests methods covering oral absorption, distribution, clearance, metabolism and specific organ- and system-toxicity, such as haemato-, immuno-, neuro-, nephro- and hepatotoxicity. The specific assays were used as alerts and correctors to identify the role of biokinetics and target-mediated toxicity for the in vitro-in vivo correlation.

Role of Absorption, Distribution & Excretion (ADE)

One of the reasons why a compound will be an outlier in the comparison between its EC_{50} value and its LD_{50} is its kinetic behaviour. Two aspects can be distinguished:

1. the kinetics of the compound in the in vitro system ('biokinetics in vitro'); and
2. the use of kinetic models in extrapolating the in vitro dose metrics to the in vivo situation.

Biokinetics in vitro

In many in vitro experiments, toxic effects or biotransformation rates are related to the concentrations of the compound added to the medium, i.e., the amount added divided by the volume of the culture medium. This nominal concentration can to a great extent deviate from the actual, free concentration of the compound in the system and also change over time, as a result of binding to proteins, binding to the culture plastic, evaporation, or uptake in the cells. Since it usually is the freely available concentration that is the driving force for toxic reactions on the (sub) cellular level, these processes will influence the free concentration and thus the effect. It is therefore necessary to estimate or measure this free concentration, especially when on the basis of the physico-chemical properties (e.g., lipophilicity) it can be expected that the free concentration will differ from the nominal concentration. One technique is to sample the culture medium with solid-phase micro-extraction (SPME) devices and to analyse the compound. These devices consist of small rods covered with material absorbing the compound in equilibrium with its free concentration. This technique allows the identification of processes influencing the free concentration. This in turn enables the modelling of the in vitro system. The application of these techniques

showed that for some compounds the free concentration could differ up to two orders of magnitude from the nominal concentration, showing the importance of understanding, measuring and modelling the 'biokinetics *in vitro*'.

However, for calculating the acute toxicity of chemicals, it became clear that only in extreme cases does this influence the estimations of the *in vivo* toxic dose, since many of the factors influencing the free concentration *in vitro* are also of influence on the free concentrations of compounds *in vivo*.

Biokinetics in in vitro-in vivo extrapolation (IVIVE)

Physiologically-based biokinetic (PBBK) models are essential tools in the evaluation of *in vitro*-derived data on dose- (or concentration)-response relationships for the situation in intact organisms.

For the purpose of predicting whether a compound would be an outlier, an evaluation of the most prominent factors for its kinetic behaviour was made. An eight-compartment PBPK model representing the main routes of elimination was used. The model describes the biokinetics of a substance after its oral uptake into the system. It was concluded that the most important parameters are:

- the extent of oral absorption of the compound
- the distribution over the tissues, as governed by its lipophilicity
- its intrinsic clearance (CLt,int)

- the free fraction (i.e., the fraction that is not bound to protein).

In WP5, the different partners have contributed *in vitro* data on these parameters, including:

- estimates of oral absorption by measuring transport over Caco-2 cell layers and over artificial membranes and by estimating the transport by use of *in silico* neural networking techniques on the basis of structural properties of the compounds under study
- estimates of metabolic stability by measuring loss of compound in different metabolising systems, including rat and human microsomes
- estimates of transport over the blood-brain barrier, by also using *in silico* neural networking techniques and by measuring the transport over *in vitro* systems representing the barrier.

On the basis of these data and considerations, the steps in the kinetic modelling of EC_{50} values for estimating LD_{50} values were developed and applied in a set of algorithms. These algorithms first took into account the calculation of the internal dose, based on the effective concentration of compounds (e.g., as EC_{50}s), the parameters for the distribution of the compound (lipophilicity, metabolic clearance, protein binding). The next step is then the calculation of the external dose (i.e., the oral absorption estimates) and the conversion from the molar dose to a dose in mg/kg bw.

The final outcome of the work in WP5 was that the corrections for the kinetic parameters led to an improvement of the correlation between *in vitro* data based on EC_{50}s for basal cytotoxicity (3T3/NRU test) with data on LD_{50}s (R^2 values increased from 0.34 to 0.55 on the inclusion of kinetic parameters). The algorithms developed were used in the prevalidation exercise. A drawback, however, was the fact that it was not possible to have *in vitro* or *in silico* data for all compounds. This was mainly due to the lack of sufficient analytical techniques.

Metabolism

New strategies to incorporate metabolic capabilities into cell lines

Recombinant-defective adenoviral vectors encoding for major CYP genes (CYP 1A2, 2A6, 2E1 and 3A4) involved in foreign compounds metabolism were transfected into hepatoma cell lines. In order to obtain functionally metabolising cells, other drug metabolising enzymes (such as cytochrome reductase, heme synthesis, etc.) must be expressed. Assay miniaturisation was also accomplished, allowing the use of 96-well plates and decrease the virus amounts necessary, thus making the process more time- and cost-effective and amenable to HTS toxicity platforms. The goal was to demonstrate the applicability of the method developed as an *in vitro* screening tool to study CYP metabolic-dependent toxicity.

Metabolic Stability of ACuteTox reference chemicals in hepatocytes & microsomes

In vitro half-life and intrinsic clearance of compounds on the ACuteTox list have been analysed, using rat liver microsomes (15 compounds analysed), human liver microsomes (32 compounds analysed), primary rat hepatocytes (15 compounds analysed), and cryopreserved human hepatocytes (21 compounds analysed). Data were expressed as *in vitro* clearance (in μl/min/mg microsomal protein or μl/min/10^6 cells) as well as calculated Clint, hepatic clearance and extraction ratio using established scaling factors. Species differences as well as large differences between the metabolic stability in the presence of liver microsomes and hepatocytes were found for some of the compounds. Data on protein binding in human plasma for 29 compounds using microdialysis and LC-MS-MS were also generated.

Evaluation of computer-based prediction models for toxicity combining in vitro data on toxicity and PBBK-TD modelling

The results obtained support the conclusion that PBBK modelling is a promising tool that is likely to improve the possibility to predict toxic doses after oral administration based on *in vitro* data, but also that it is reasonable to question the approach of using only basal cytotoxicity data for performing such predictions. Recalculations were made using the permeability converter included in the computational model GastroPlus™.

Computer-based prediction of metabolism & integration of metabolism data into toxicity screening

Major metabolites of a number of test compounds (amiodarone, acetaminophen, acetylsalicylic acid, atropine, caffeine, carbamazepine, colchicine, cycloheximide, diazepam, nicotine, orphenadrine, phenobarbital, valproate and verapamil) identified *in vitro* by LC-MS-MS or found in the literature (*in vivo* or *in vitro* data) were compared to metabolites predicted by the METEOR software at different levels of probability. METEOR was found to predict most of the major metabolites (81%). In 7 out of 14 compounds all major metabolites were predicted correctly.

The toxicity of 17 substances from the ACuteTox programme was predicted by the DEREK predictive software. For twelve of these substances, one or several DEREK alerts were obtained. Four of the substances where not flagged at all by the software. For one of these substances, carbamazepine, a possibly toxic metabolite carbamazepine epoxide was predicted by METEOR (confirmed *in vivo*). When a prediction for this metabolite was performed, several DEREK alerts were obtained. It is concluded that the combined use of DEREK and METEOR is likely to improve the possibility to predict the toxicity of an unknown substance and or its major metabolites. Further analysis needs to be performed before any general conclusion regarding the usefulness of DEREK for this type of analysis can be made.

A preliminary investigation on the possibility to use DEREK for ranking of compounds regarding their potential to induce acute toxicity has been performed. The rationale for doing this, although acute toxicity is not an established endpoint included in DEREK, is that compounds that have the potential to induce acute toxicity (at least compounds being potent enough) might be expected also to induce one or more different types of subchronic or chronic toxicities at lower doses. It was found that nearly all substances in the dataset used (482 substances) that obtained one or more DEREK 'hits' (310 compounds) had an oral LD_{50} dose close to or below 100 mg/kg. Only 2 of the compounds with an oral LD_{50} above 100 mg/kg (N-hexane and butyric acid) obtained any flag (both compounds received 1 flag). However, since only 16 of the compounds in the dataset had an oral LD_{50} above 100 mg/kg, further analysis are needed before any general conclusions can be made.

Hepatotoxicity

Integrated strategy to alert about hepatotoxic (intrinsic &/or bioactivable) compounds, as a part of a general AcuteTox testing platform

The scientific purpose was to set up an integrated strategy to alert about intrinsic hepatotoxic compounds, as well as metabolism-dependent toxicity (bioactivation) by assaying cytotoxicity on three cell systems. The idea of generating 'alerts' for warning about a possible deviation of a compound in the

3T3/NRU cytotoxicity test, rather than assessing potential hepatotoxicity, has prevailed in the final decision of adopting a simple, yet mechanistically-based test. The issue addressed was whether such a cell-based test would be capable of discriminating bioactivable compounds as well compounds showing a preferential action on hepatocytes, i.e.:

1. Some compounds may elicit preferential toxicity on hepatic cells (hepatocytes and HepG2) in comparison with the non-hepatic cell line 3T3, indicating that such compound affects hepatic cells without requiring biotransformation;
2. A bioactivable compound is expected to cause more toxicity in hepatocytes than in non-metabolising cells (HepG2 and 3T3);
3. Finally, the compound may show similar toxicities in the three cell types, indicating that it primarily exerts basal cytotoxicity.

The SOPs for cell cultures, as well as for MTT assay as end-point for basal cytotoxicity, were used by all the partners of WP6 and WP7.3. ECOD activity was determined in rat hepatocyte cultures as quality control criteria.

Sensitivity, reproducibility, robustness & potential transferability of the assay

To examine the robustness of the strategy, the intra-assay, inter-assay as well as the intra-laboratory variability was evaluated for each cell system in each laboratory. A low variability (% CV < 10%), both intra-plate and intra-assay, was obtained in all

laboratories. However, the analysis of the first 21 compounds showed that the intra-laboratory variability needs to be reduced. A similar intra-laboratory variability and the lack of reproducibility of the cytotoxicity data was found with the second set of 41 compounds. The variability in HepG2 found in the assay run in a robotic system was very similar to that of the other partners, thus, manipulation seems not to be the main cause of the observed intra-laboratory variability. In addition, broad inter-laboratory variability (% CV of IC_{50}) has been found in the 5 participant laboratories in WP6 and WP7.3, both in the 21-first and in the 41-second set of compounds, despite the fact that all labs used the same cell models and the same SOPs. Other factors responsible for this intra- and inter-laboratory variability were further examined.

The independent statistical analysis of the data shows that the 3T3/MTT and the HepG2/MTT (with the exception of a few outlying CVs larger 60%) assays show similar variability as 3T3/NRU cytotoxicity assay, while control response variability is apparently larger for the primary rat hepatocytes/MTT assay. Variability of control response was investigated also separately for every partner per cell line combination. Different variability in control response was observed for different partners, and variability differed between the three cell lines. The analysis of the variation (% CV) of EC_{50} estimates of the reference chemicals (SLS, amiodarone and sodium valproate) clearly differs between the partners. Additionally, comparison of EC_{50} estimations for the

three reference compounds revealed that the quality of the results obtained in the primary rat hepatocytes/MTT assay was highly variable among partners.

Predictability/ indication of alert of bioactivation &/ or hepatotoxicity

The cytotoxicity was low (IC50 > 1E-03 M) for 25 of the tested compounds. For 13 compounds, IC50s were higher than the highest soluble concentration of the compounds in culture medium (incomplete concentration-effect curves). In these cases, the MRTE value calculated by the software was used in the comparison of all the curves in the analysis of the data. First cytotoxicity in heptocytes and HepG2 (WP6) were compared to alert bioactivation and thereafter hepatocytes, Hep G2 and 3T3 were compared to alert bioactivation/hepatotoxicity.

In view of the high % CV in the data obtained in all cellular models, the analysis of variance (ANOVA) does not identify statistical significant differences in the comparisons among different cells lines for a huge number of chemicals making it very hard to identify alerts of hepatotoxicity and/or bioactivation.

Hepatotoxicity alert

Some hepatotoxic compounds (acetaminophen, acetylsalicylic acid, tetracycline hydrochloride, verapamil hydrochloride, chlorpromazine, rifampicine, orphenadrine hydrochloride and parathion) showed very high % CV in all cellular models and were classified as

undefined). Other hepatotoxic compounds (classified as cytotoxic): ethanol, sodium valproate and amiodarone (reported steatosic), chlorpromazine, cyclosporine A and 17α-ethynylestradiol (reported cholestatic), pentachlorophenol and lindane (reported genotoxic), parathion (reported mainly neurotoxic) were not alerted, likely due to the fact that these toxic effects are chronic and not acute. Cyclophosphamide (low toxicity $IC_{50} > 1E-03$ M) and carbamazepine was not alerted, while tamoxifen and acrylaldehyde were correctly alerted as hepatotoxic.

Bioactivation alert

Ten compounds reported as bioactivable in the literature were analysed. Acetaminophen and verapamil showed very high % CV in all cellular models and are classified as undefined. Several reasons justify that certain compounds were not alerted as bioactivable: orphenadrine hydrochloride (very high % CV in all cellular models), tamoxifen (data reported in human liver/hepatocytes), carbamazepine, cyclophosphamide (low toxicity, $IC_{50} > 1E-03$ M), pentachlorophenol (reported genotoxic), sodium valproate (reported steatosic), and 17α-ethynylestradiol (reported cholestatic). Therefore, these compounds were not alerted, likely due to the fact that these toxic effects are chronic and not acute. However, amphetamine sulphate reported in the literature as bioactivable was correctly alerted in the assay.

Cytotoxic compounds

No difference was obtained in cytotoxicity among the three cellular models for the rest of compounds; any of them were reported as hepatotoxic and/or bioactivable, and were therefore correctly classified as cytotoxic.

Criteria for alerting hepatotoxicity

The results suggest that only if intra-laboratory data (indeed inter-laboratory) for a given chemical are reproducible (% CV < 15%) in all cellular models (especially for the reference compounds), the software may deliver consistent information for both ICs and MRTE. Consequently the ANOVA can identify statistically significant differences in the comparisons among different cell systems allowing identification of reliable alerts of hepatotoxicity and/or bioactivation. The last part of the analysis is based on the comparison of the cells using the cut-off configured by the user in order to know which cell type shows higher toxicity or if all of them are similar. Tentatively, cut-off values for IC_{50} ratios >2 have been proposed to alert on bioactivation or hepatotoxicity; lower ratios are considered not reliable. When compounds elicited very weak toxicities ($IC_{50} \geq$ 1E-02 M), the ratio of IC_{50} values between pairs of cell models should be $\geq$ 5 to be considered an 'alert'.

Haematotoxicity

The human whole blood assay measuring cytokine release

The multiplexed assay is based upon the use of a dedicated flow cytometer (Luminex system) and a mixture of fluorescent microspheres covered with capture antibodies that bind specific analytes; in this case, the inflammatory cytokines IFN-g, TNF-α and IL-6 from the supernatants of cell cultures. Bound cytokines are, in turn, revealed by a second fluorescent antibody. In this way, the concentration of several cytokines (multiplexing) can be determined simultaneously when bead mixtures are run through the flow cytometer and

Table 2. The optimised neurotoxicity test battery.

Endpoints	In Vitro Model
GABAA receptor function	Primary cortical mouse neurons
Cell membrane potential	Neuroblastoma cell line, SH-SY5Y
Acetylcholine esterase activity	Neuroblastoma cell line, SH-SY5Y
Transcriptional markers (NF-H, GFAP, MBP, HSP32)	Aggregated rat brain cells
Metabolic markers (glucose uptake, mRNA synthesis)	Aggregated rat brain cells
Caspase-3 mRNA expression	Primary rat cerebellar granule cells

a calibration curve constructed. The assay involves incubation of cultures of human whole blood for 24-hours with lipopolysaccharide (LPS), a monocyte activator, in the presence or absence of a range of concentrations test compounds or appropriate immunosuppresors. Endpoint measured is secretion of IFN-g, TNF-a and IL-6 and the expression of the final results is given by IC_{50} (inhibition by test compound of LPS-induced cytokine secretion) or EC_{50} (enhancement by test compound of LPS-induced cytokine secretion).

The Colony forming unit-granulocytes/ macrophage assay (CFU-GM)

Human umbilical cord blood cells were mixed in tubes containing cell culture mixture with linearity controls, vehicle controls, and eight concentrations of reference compounds for the dose-response curve. Each tube was used to prepare three culture dishes. All the toxicant dilutions were prepared at 200x the final dilution in order to obtain the final fold-dilutions of drug in the culture dish. The cultures were incubated at 37°C in air + 5% CO_2 under saturated humidity for 14 days. All dishes were scored for colony counts following a random fashion. The survival curves of CFU-GMs exposed to different doses of the compounds were obtained and IC values were calculated.

Combinatory 'New' Endpoints

The Cytomics Toxicity Panel

This group of assays is based on the use of flow cytometry and endpoint-specific fluorescent probes for general cytotoxicity markers. By means of the multiparametric capability of cytomics, measurements are restricted to live cells, thus providing early biomarkers of cytotoxicity, evident before the cell is displaying an overt death programme. The cell system used were human established cell lines of different (neuroblastoma SH-SY5Y, hepatoma HepG2 and kidney adenocarcinoma A704). Cells were exposed for 24-hours (SH-SY5Y and HepG2 cell lines) or 48-hours (A704 cells), and a range of 3 to 4 concentrations tested. The assay panel measures the following endpoints: intracellular Ca^{2+}, plasma membrane potential, and mitochondrial membrane potential. The expression of the final results was done either as IC_{50} (decreased intensity of endpoint-associated fluorescence) or EC_{50} (increased intensity of endpoint-associated fluorescence) values.

The Cytomics Oxidative Stress Panel

This group of assays is based on the use of flow cytometry and high-content analysis by bioimaging to quantify endpoint-specific fluorescent probes for oxidative stress markers. By means of the multiparametric capability of cytomics, measurements are restricted to live cells, thus providing early biomarkers of oxidative damage to cells, evident before the cell is displaying an overt death programme. The cell systems used were human established cell lines of different origin (neuroblastoma SH-SY5Y, hepatoma HepG2 and kidney adenocarcinoma A704). Cells were exposed for 24 hours (SH-SY5Y and HepG2

Table 3. The best performing in vitro assays, which were selected as candidates for the final tiered testing strategy, and further evaluated in the prevalidation phase.

Selected Assay	Target (WP Involved)
The neutral red uptake assay using the 3T3 fibroblast cell line (3T3/NRU)	General basal cytotoxicity (WP2)
The cytokine release assay using human whole blood (IL-1, IL-6, TNF-α)	Haemotoxicity (WP4)
Cell differentiation in human cord blood-derived cells (CBC/CFU-GM)	Haemotoxicity (WP4)
Gene expression (GFAP, HSP-32, MBP & NF-H) in primary rat brain aggregate cultures	Neurotoxicity (WP7.1)
Uridine incorporation measuring the total mRNA synthesis in primary rat brain aggregate cultures	Neurotoxicity (WP7.1)
Cytomic panel measuring oxidative stress (intracellular peroxidative activity, intracellular levels of superoxide anion, oxidised DNA base 8-oxoguanine) in HepG2, SH-SY5Y and A.704 cells	New endpoints (WP4)
Cytomic panel for cytotoxicity screening (intracellular Ca2+ levels, mitochondrial membrane potential, plasma membrane potential) in HepG2, SH-SY5Y and A.704 cells	New endpoints (WP4)
The MTT assay using primary rat hepatocytes	Metabolism (WP6)
Kinetic parameters: volume of distribution, protein binding, clearance & oral absorption (Caco-2 cells) for the estimation of the oral dose from the effective concentration observed *in vitro*	Biokinetics (WP5)
The estimation of compound passage through the blood-brain barrier using neuronal networks (for neurotoxicity assays)	Biokinetics (WP5)

cell lines) or 48 hours (A704 cells) and a range of 3 to 4 concentrations tested. The assay panel measures the following endpoints: mitochondrial superoxide anion and intracellular peroxidative activity by flow cytometry and the levels of oxidised DNA base 2-deoxy-8-guanine in genomic DNA and in mitochondrial DNA by high-content analysis by bioimaging. The expression of the final results was done either as IC_{50} (decreased intensity of endpoint-associated fluorescence) or EC_{50} (increased intensity of endpoint-associated fluorescence) values.

Neurotoxicity

As a first screening for the most useful neurotoxicity assays, 16 general reference chemicals plus the 7 reference chemicals with neurotoxic potential were first tested 43 neurotoxicity assays. Some methods could determine selective effects on receptor and ion channel function, as well as neurotransmitter synthesis, release, degradation and uptake. We analysed genomic expression of approximately 40 proteins by real-time quantitative RT-PCR. The cell membrane potential, mitochondrial membrane potential, ATP synthesis, ROS production, global glucose uptake and protein- and RNA synthesis which are crucial for neuronal function, were also investigated. For comparison, short- and long-term general cytotoxicity analyses were performed in different neuronal cell systems.

Lowest observed effective concentrations (LOEC), EC_{20} and EC_{50} values (i.e., neurotoxic concentrations, NTC) were

determined from each assay for the 23 reference chemicals and the values were compared with IC_{50} generated by the general cytotoxicity 3T3/NRU assay. Neurotoxic concentrations which were 0.7 log units lower than the cytotoxic IC_{50} indicated potential neurotoxicity, i.e., alerting acute systemic toxicity by a neurotoxic mechanism. The possibility to correct underestimated toxicity by substituting cytotoxic IC_{50} values with the neurotoxic concentrations was also used as an indication of the predictive capacity of the neurotoxicity endpoints. Both criteria indicated a subset of assays that identified the most neurotoxic alerts and also had the best correction capacity (Table 2).

Results of the extended testing using the optimised neurotoxicity test battery

All outliers identified in the correlation between *in vivo* toxicity data, i.e., human lethal blood concentration (LC_{50}) and LD_{50} in rats, and *in vitro* cytotoxicity, i.e., IC_{50} determined using the 3T3/NRU assay could be identified as potentially neurotoxic by one or more assay in the optimised neurotoxicity test battery, except for acetaminophen and thallium sulphate. Acetaminophen is well known to induce liver toxicity and is not expected to be identified as a neurotoxic compound. Thallium sulphate accumulates in body tissues after *in vivo* exposure, which may result in increasing concentration in the target tissue with time. Hence, the identification of thallium sulphate as an outlier in the in IC_{50} vs. LD_{50} correlation as well as the failure to alert for neurotoxicity may be due to toxicokinetic factors.

The combinatory assay analysing transcriptional gene expression in aggregated rat brain cells alerted as many as 45% of the test chemicals as neurotoxic and severe outliers. The assays analysing cell membrane potential, $GABA_A$ receptor function, acetylcholine esterase activity and caspase-3 expression identified additional neurotoxic chemicals, which could not be detected by the endpoints analysed in rat brain cell cultures. Taking other issues into account such as variability and limitations, the multi-endpoint aggregate assay was the most promising neurotoxicity assay to be integrated into the test strategy for identification of acutely toxic chemicals (see below). However, it must be remembered that the blood-brain barrier may hinder chemicals to enter the brain parenchyma from blood, which means that the neurotoxic concentrations determined by the assays presented herein may be significantly higher as compared to the lethal blood concentrations.

Nephrotoxicity

For the measurement of nephrotoxicity, transepithelial resistance (TER) was chosen as the functional assay and the LLC-PK1 proximal tubular cell line as the test system. The functional assay reflects the *in vivo* transporting capabilities of the renal proximal tubules. The functional assay was compared to a viability assay namely the resazurin ('Alamar blue') assay under exactly the same experimental conditions. Fourteen chemicals were analysed in the two assays but in two different laboratories. The inter-laboratory comparison of the IC_{50} values for TER and resazurin assays showed very good correlation and the REMS automated device facilitates high-throughput of the TER assay. Furthermore, two set-ups of the test system were evaluated and compared; a 24-well Costar HTS polycarbonate filter plate system and a 96-well system, which showed good correlation between the two systems. Hence, the REMS automated device with the 96-well system was selected for measurement of TER.

The 57 reference chemicals (including some nephrotoxic) were tested and the overall results show that the TER is a sensitive predictor of nephrotoxicity. Using the data obtained, IC_{20}, IC_{50} and IC_{80} values were calculated for all chemicals tested. TER showed greater sensitivity for nephrotoxic chemicals compared to non-nephrotoxic chemicals. However, compounds requiring metabolism, such as diethylene glycol, did not show toxicity at the highest concentration tested. The results indicate that the TER functional assay is a very promising assay to detect nephrotoxicity *in vitro* and is more sensitive than the viability assay.

In terms of alerts, all the known nephrotoxins had an IC_{50} value of less than 50 µM in the TER assay. For the non-nephrotoxic reference compounds, this property was only shared by digoxin, where one of the mechanisms of action involves inhibition of the Na^+/K^+ pump. However, the TER assay was not finally selected in the tiered testing strategy in comparison to the 3T3 fibroblast cell line viability assay (3T3/NRU) because the

multivariate CART results did not change after removing TER from multivariate CART models (see below). This result may be due to the fact that the list of reference chemicals contained only few examples of pure nephrotoxins which did not affect other organ model systems.

Statistical Analyses & Optimisation of Strategy Models for Classification

The selection of the *in vitro/in silico* methods for the prevalidation exercise from the total number of assays performed in the ACuteTox project was based on an analysis of variability, repeatability and reproducibility of the single assays, as well as the assessment of preliminary predictive capacity using univariate and multivariate CART analyses.

The analysis consisted in the statistical evaluation of the concentration-response curves and the computation of EC_{20}, EC_{50} or LOEC values for every experiment performed. The assays were also compared with respect to their repeatability and reproducibility. Furthermore, an overview on the bivariate dependency between different assays was shown.

The outcome of this first evaluation resulted in the exclusion of some assays from further analysis due to their insufficient performance, to lack of data for all 56 chemicals or to other reasons (e.g., many uncensored values obtained with an assay, failure to identify positive compounds).

Since the ultimate aim of the statistical analysis for ACuteTox is the classification of chemicals into the official acute oral toxicity categories (EU and GHS), the Classification and Regression Trees (CART) was used as the classification algorithm of choice. After derivation of a CART tree, misclassification rate was estimated from the data. An unbiased estimate of the misclassification rate was obtained from the application of the CART tree to independent data, such as the data of the prevalidation that involved testing of a new set of chemicals.

CART analyses were performed with untransformed EC_{20}, EC_{50} or LOEC values for the *in vitro* assays. In addition, for the neurotoxicity assays, a blood brain barrier (BBB) transformation was performed and another approach was used in which the calculated EC_{20}, EC_{50} and LOEC values from the *in vitro* assays were first transformed to rat LD_{50} values using the transformation suggested by WP5.

The overall analysis performed before the start of the prevalidation exercise resulted in the selection of 8 methods (Table 3) and the proposal of different combinations of the selected assays (i.e., testing strategies). The proposed combinations were challenged with the new data generated during the prevalidation in order to identify the best performing strategy.

The work performed during the prevalidation focused mainly on the assessment of the predictive capacity of the proposed tiered testing strategies and the identification of the combination of

methods that gave the best prediction, in terms of classification of compounds in the official acute oral toxicity categories.

During this prevalidation study, the methods identified as promising building blocks for the testing strategy on the basis of the so-called training set of compounds (57 previously tested compounds) were challenged under blind conditions with a new set of 32 test compounds. The use of reference compounds not included in the training set was essential in order to properly assess the predictive capacity of the tiered testing strategy.

Due to time constrains the assessment of transferability of the methods to a second independent (naïve) laboratory was not feasible, as it requires extensive training of the naïve laboratory. Therefore, laboratories that were involved in the development and optimisation of the selected test methods, took part in the testing exercise.

The evaluation of raw data was performed in the same manner as for the optimisation phase of the project. In addition to the CART methodology used in the first phase of the project, the Random Forest model was used for the classification task during the prevalidation. Two classification approaches were studied in detail: single-step procedures and two-step tiered testing strategies. The strategy that uses the Random Forest model, including 7 assays in a single step procedure, gave the best correct classification rate (69.3%) and resulted in only 2 compounds with underestimated toxicity (brucine,

paraquat), as compared to the official acute oral toxicity classification.

However, the overall results showed that the incorporation of additional endpoints did not improve significantly the outcome of the 3T3/NRU cytotoxicity assay alone in terms of classification of compounds for acute oral toxicity. Only compounds with LD_{50} > 2000 mg/kg are best classified, while the other toxicity categories are misclassified.

As shown in WP7, a number of assays were identified that were able to flag compounds as neurotoxicants and nephrotoxicants, both in the training (57 compounds) and test sets (32 compounds). Therefore, those *in vitro* assays could be used to alert on tissue specific toxicity for compounds that are identified as toxic (predicted LD_{50} < 2000 mg/kg) with the 3T3/NRU assay.

Challenges & Solutions

The results obtained from the classification analysis performed in the ACuteTox project led us to question the scientific motivation for the current classification systems for acute oral toxicity that are based on arbitrary cut-off for rat oral LD_{50} values, and to suggest the revision of the GHS/ EU CLP systems. The outcome of the analysis of consistency in classification (GHS and EU categories) corresponding to the reported ranges of LD_{50} respective of individual 97 substances included in the ACuteTox project give an indication of potential consequence for ambiguity in corresponding classification. The analysis showed that (with at least 90%

probability) ~50% of the substances would be unequivocally classified by a single category, ~40% would ambiguously occur within the limits of two adjacent classification categories, and ~10% of the substances have LD_{50} ranges of sufficient scope to span three or more different classifications. This analysis reinforced previous findings by Rudén and Hansson[5] and leads to the same recommendation of revision of the GHS/CLP system.

The estimation of the oral dose by including kinetic parameters needs to be further evaluated, in particular the availability of well established in house validated analytical methods for non-drug like compounds is at present a limiting factor, and requires further investment in future strategies. If more data on kinetic parameters were available, a better evaluation of the impact of the different kinetic factors, i.e., absorption, distribution (e.g., lipophilicity, protein binding) needs to be made.

Concluding Remarks

In the ACuteTox project undertook for the first time a challenging goal to create an integrated testing strategy to replace the animal testing used today for predicting human acute oral systemic toxicity based exclusively on *in vitro* and *in silico* methods.

This project represents also the first attempt to pre-validate a testing strategy

based exclusively on non-animal methods, and therefore provides an excellent case study for ECVAM and helps progressing the discussions on validation of testing strategies which are currently ongoing at several levels, i.e., ECVAM, European Partnership for Alternative Approaches to Animal Testing (EPAA), COLIPA, ECHA and others.

In the first phase of the project, a very large number of *in vitro* test methods (approximately 75 endpoints) have been evaluated in terms of their within-laboratory variability (and in some cases also the between-laboratory variability), preliminary predictive capacity and the potential to identify alerts for organ-specific toxicity, The outcome of this phase of the project is a large toolbox of *in vitro* methods with associated optimised protocols, some of them evaluated to the level of prevalidation.

An in-depth statistical analysis of the large dataset generated in this project resulted in a list of 8 *in vitro* and *in silico* methods, which resulted to be the most promising for inclusion in proposal of potential testing strategies. Protocols of all these methods will be available to the public as INVITTOX protocols, through the ECVAM database on alternative methods (DB-ALM).

The last phase of the ACuteTox project focused mainly on the assessment of the predictive capacity of the proposed tiered testing strategies and the identification of assay combinations that give the best prediction in terms of classifying

[5] Rudén C, Hansson SO. How accurate are the European Union's classifications of chemical substances. Toxicol Lett. 2003; 144, 159-72.

chemicals into the official acute oral toxicity categories (GHS and EU CLP systems). Five proposals for *in vitro* tiered testing strategies were formulated and evaluated in terms of predictivity.

The outcome of this analysis reinforced previous results obtained with the 3T3/NRU assays and supports the use of this validated cytotoxicity assay to identify unclassified substances (LD_{50} > 2000 mg/kg), as a first step in a tiered testing strategy.

Several *in vitro* assays have proved to be useful to identify alerts for tissue specific toxicities such as neurotoxicity and nephrotoxicity. However, the results of the classification analysis showed that complementing the 3T3/NRU assay with those *in vitro* assays is not improving significantly the classification of compounds in toxicity categories 1-4. This outcome is largely linked to the fact that the current classification systems are based on arbitrarily assigned cut-offs for the rat LD_{50} values, and do not include more detailed scientific (mechanistic) information on the compounds. Thus, a revision of the current classifications schemes might be advisable and should be put forward to the European regulatory agencies.

Most of the results obtained in the course of the ACuteTox project have resulted in peer-reviewed publications, which proves the scientific quality of the data generated. The dissemination activities will continue, and in particular a special issue in *Toxicology In Vitro* is under preparation.

Some of the knowledge derived from the project has been already applied in the daily activities of companies that are ACuteTox partners (e.g., pharmaceutical sector). This goes beyond the main objective of the ACuteTox project (prediction of acute oral toxicity) and helps improving European competitiveness. Examples include, for instance, the case of Noscira, which increased the confidence in the results obtained by using some of the neurotoxicity models that were optimised during the project.

In the same direction, the project allowed the establishment of interesting specific collaborations with individual partners covering, e.g., aspects of hepatotoxicity and metabolism, not only at the experimental level, but also in the preparation of dossiers and documents being presented to both the European Medicines Agency and the US Food and Drug Administration, which in the end is a definitive success of the project.

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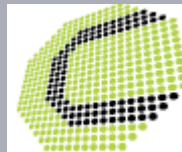Uppsala, Sweden

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carcinoGENOMICS

Development of a High-Throughput Genomics-Based Test for Assessing Genotoxic & Carcinogenic Properties of Chemical Compounds *In Vitro*

Contract number: LSHB-CT-2004-512051
Project type: Integrated Project (FP6)
EC contribution: € 10,440,000
Starting date: 1 November 2006
Duration: 60 months
Website: carcinogenomics.eu

Background & Objectives

carcinoGENOMICS is an FP6 Integrated Project with 20 partners across Europe that is devoted to developing *in vitro* methods for assessing the carcinogenic potential of compounds as an alternative to current rodent bioassays for genotoxicity and carcinogenicity.

The carcinoGENOMICS project set out to develop a battery of mechanism-based *in vitro* tests accounting for various modes of carcinogenic action. These tests have been designed to cover major target organs for carcinogenic action, e.g., liver, lung and kidney. The novel assays that have now become available in the fourth year of the project are based on the application of 'omics' technologies (i.e., genome-wide transcriptomics as well as metabonomics) to robust *in vitro* systems applying human cellular models (for which also embryonic stem cell technology has been explored) to generate 'omic' responses from a well-defined set of model compounds causing genotoxicity and carcinogenicity. For selecting the most promising models, phenotypic markers for genotoxic and carcinogenic events have also been assessed for the purpose of anchoring gene expression modulations, metabolic profiles and mechanistic pathways. Through extensive biostatistics, literature mining and analysis of molecular-expression datasets, differential genetic pathways were identified capable of predicting mechanisms of chemical carcinogenesis *in vivo*. It is thus expected that the final outcome of this project will generate a platform enabling the investigation of large numbers of compounds for

their genotoxic and carcinogenic potential, as envisaged under the EU 'REACH' chemicals regulation. This will contribute to speeding the identification of potential harmful substances to humans, while lowering costs and reducing animal tests.

The research hypothesis underlying carcinoGENOMICS is that it is feasible to generate transcriptomic and metabonomic profiles from a set of well-defined genotoxic and non-genotoxic carcinogenic compounds in *in vitro* cellular systems that reliably predict genotoxic and carcinogenic events *in vivo*. To evaluate this hypothesis, the project has been comprised of different components in such a way that the latest innovations in cell technology, genomics analysis, and bioinformatics will be fully exploited:

- The first component refers to the innovative cell technology to be used. Current *in vitro* models representing epithelial target organs such as liver, kidney and lung, are criticised in that they generally rely on (tumour) cell lines, which may strongly differ in their functional characteristics from normal cells. In addition, they show considerably less metabolic activity than differentiated cells, which is of utmost relevance as most chemical carcinogens require bioactivation before eliciting their toxic effect;
- The second component refers to the novel combination of transcriptomic and metabonomic analyses of carcinogen-exposed cellular systems. Both transcriptomics and metabonomics are established

genomic technologies, which have been demonstrated to yield profiles capable of discriminating classes of chemical agents. It is hypothesised that through combining transcriptomic and metabonomic data sets as foreseen in this project, predictive values of generated profiles will be endorsed;
- The third component refers to novel applications of bioinformatics, with respect to standardising the infrastructure for data storage and mining at high-quality level.

Therefore, the carcinoGENOMICS project has particularly focused on the development of new *in vitro tests* to replace animal experimentation. The project specifically aims at developing robust and effective 'omics-based cellular models representing target organs for carcinogenic actions *in vivo* that can be applied as alternatives for reducing the need of animal tests and eventually for replacing current rodent assays for assessing genotoxic and carcinogenic features of chemicals. Its main target thus will be replacing current bioassays for *in vivo* genotoxicity and carcinogenicity, which have limited biological plausibility while being threatening to animal welfare, costly, laborious and time-consuming; therefore, the project strongly complies with policies regarding the protection of animals used for experimental and other scientific purposes. Additionally, it is envisaged that the *in vitro* assays to be developed under carcinoGENOMICS will outperform the current *in vitro* mammalian cell genotoxicity assays,

which are hampered by their very limited predictiveness and their high rate of false-positives.

In general, it is important to note that the detailed characterisation of any experimental model is essential since it will allow for establishing guidelines for use of developed assays in cancer safety risk assessment. In addition to characterisation, defined criteria for quantifying the performance and validating the biological performance of any experimental model are strongly encouraged. From the beginning of the carcinoGENOMICS project, several *in vitro* models per target organ have been considered. Further characterisations of these models, with major attention for metabolic competences and physiological features, have been performed. Representative *in vitro* models have thereupon been selected, and during the fourth year of this project, a first series of challenges of these models by selected carcinogens and noncarcinogens for generating 'omics as well as functional responses have been performed. Initial data analysis had been applied. The data warehousing infrastructure has also been completed. And in the area of developing computerised models, major efforts have been undertaken over the past year, in particular concerning the modelling of apoptotic responses. Very importantly, a major milestone had been reached during the fourth year (month 44), i.e., the final selection of one cellular model per target organ, thus ending Phase I of this project.

During the fourth year, training efforts within carcinoGENOMICS has been focused on practical trainings organised by the consortium. Within the carcinoGENOMICS project, large transcriptome data sets will be generated in order to gain a genome-wide view of the response of cellular systems on compound treatment. Therefore, as a sequel to earlier training on RNA isolation, safe handling of carcinogenic substances, metabonomics data analysis, and practical trainings for junior scientists on the presentation of scientific data, bioinformatics workshops have been organised in the past year to introduce the tools and statistical methods available to analyse the data that will be generated under this project. The main goal of these workshops was to gain a basic understanding of the underlying methodologies, of the questions that can be solved by these methods, and of the interpretation of the results. Methods covered the primary analysis of case-control and multiple-case studies, as well as higher-level of data analysis.

WP1: Selection of Chemicals

Although being closed at the end of year 3, considerable changes have been made to the set of compounds for the second phase of the project over the course of year 4. In particular, and upon specific request by the scientific advisory board, the second list of chemicals has been more 'humanised', i.e., compounds with known activity in humans have been included in the list. Compounds listed by IARC were prioritised in this respect. The modified list of compounds was formally approved by the consortium during the

Table 1. Overview of carcinoGENOMICS deliverables as achieved during the months M37-48.

Del. No.	Deliverable Name	Delivery Date	Completed	Remark
D11.2.7	Report on newsletter	M37	√	
D12.19	Annual report to the Commission: updated Description of Work	M38	√	
D12.20	Annual report to the Commission: Periodic Management Report	M38	√	
D12.21	Annual report to the Commission: Periodic Activity Report	M38	√	
D12.22	3 monthly Project Board mtg	M39	√	
D3.9	Identity of most suitable cytomic assays of relevance to carcinogenicity that can be adapted to a relatively high-throughput in our cell systems	M40	Postponed to M59	An interim deliverable being submitted & will be fully completed in the final year. Final report postponed to M59
D2.1.10	Toxicity assessment of the new set of chemicals in selected liver *in vitro* models	M42	√	
D2.3.1	A list of carcinogens & MOELs (Minimal Observed Effect Level) for their toxic effects in human liver slices from 5 donors	M42	√	
D4.6	Data allowing for the identification of novel early markers for carcinogenesis available for analysis	M42	Still ongoing	

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	Analysis was performed for 6 compounds & promising classifiers (assumed to be 'early') were identified discriminating genotoxic from non-genotoxic compounds & non-carcinogens; more compounds were tested & the analysis of the data is in process			
D5.2	Genome wide expression profiles induced by the genotoxic & carcinogenic compounds in the various *in vitro* models	M42	Postponed	
M55	Continuously genome-wide expression profiles induced by the genotoxic & carcinogenic compounds in the various *in vitro* models are measured by DNA microarrays; up to Nov. 2010 this has been done for >2000 RNA samples			
D6.6	Metabolic profiles for further sets of samples generated by WP2-4	M42	√	
D7.3.6.1	Meta-analysis results on project data - report	M42	√	
D7.3.7.1	Classification analysis - report	M42	√	
D9.2	Detailed revised SOP for each method	M42	√	
D11.1.5	Agenda for capacity building meetings	M42	√	

D12.23	3 monthly Project Board mtg	M42	√	
D11.2.8	Report on the newsletter	M43	√	
D2.2.10	Carcinogenicity testing with compounds from the extended list	M44	√	
D3.8	Comparison of dose-response relationships to selected carcinogens in cell models with & without introduction of xenobiotic metabolising capability	M44	√	
D4.5	A procedure based upon immortalised primary human lung cells for screening carcinogens	M44	Still ongoing	This procedure seems to be established, but performance of the cell line as compared to the primary cell remains to be established
D7.1.6	Report on testing and training phase & local installation deployed	M44	√	
D7.1.7	Report of consortium datasets, annotated & loaded in the infrastructure	M44	√	
D9.1	A list of promising methods to enter pre-validation	M44	√	
D9.3	A list of chemicals for pre-validation	M44	√	
D9.4	Kick-off pre-validation meeting with participating labs	M44	√	
D11.1.6	Report of the invitational conference on risk assessment	M44	√	

D11.1.8	Production of a final version manuscript for publication out of Workshop M20	M44	√	
D2.3.7	Incubation of 'risky phenotype' HepG2 with selected classic bio-activable carcinogens; cytotoxicity assessment	M45	√	
D12.24	3 monthly Project Board mtg	M45	√	
D7.2.11	Completed 4th analysis of a limited selected transcript-omics & metabolomics dataset within pathway context	M46	√	
D2.2.11	A feeder-free protocol to derive hepatocyte-like cells from hESC	M47	√	
D7.2.10	Report on completed 4th analysis of limited transcriptomics & metabolomics data-set within pathway context, provided sufficient data are generated in the con-sortium to justify com-pilation of 2nd analysis (otherwise only Delive-rable D7.2.3 will stand)	M47	√	
D4.9	Report on the Epithelix tests & decision	M48	√	
D6.7	Multivariate statistical models of further sets of metabonomic data	M48	√	
D7.3.5D	Modelling of carcinogenic effects of compounds - report	M48	√	

annual carcinoGENOMICS meeting in November 2010.

WP2: Liver Models

- *WP2.1 Improvement of current liver cellular systems:* Based on extensive 'omics data generated from (non-) carcinogen-challenged liver cellular models analysed via multiple statistical evaluations, WP2.1 was capable of selecting the HepaRG cell line as the best liver *in vitro* model.
- *WP2.2 Stem Cell Technology:* Cellartis has been able to establish robust protocols to derive hepatocyte-like cells from human embryonic stem cells (hESC). With these derived hepatocyte-like cells it appeared possible to perform and deliver carcinogenicity testing of all Phase I compounds selected by WP1. Analyses of the generated global gene expression data show that this cell model has a potential to discriminate between compound classes and could have a role in predication of carcinogenicity. The hESC model performed only slightly less than the ultimately selected HepaRG model.
- In parallel, Cellartis has been working on and planning for further scaling-up of the cell production. Extensive work has been carried out in order to modify and adapt the hep protocols to feeder-free cultured hESC. Cellartis has established a protocol to derive feeder-free cultured hESC to hepatocyte-like cells with high reproducibility. With this feeder-free system hepatocyte-like cells can be produced with a

significantly improved scalability and to a lower cost. This will lead to more efficient and cheaper studies in the future.

WP3: Kidney Models

In year 4, a range of possibilities to introduce xenobiotic metabolising capability into the renal cell systems has been explored. The use of microsomes or S9 fractions appears to be the most promising. However, it should be noted the gene array experiments indicate that in our cell systems either human RPTEC/TERT1 or rat NRK-52E, compounds traditionally regarded as requiring metabolism (streptozotocin and benzo[a]pyrene), elicited a carcinogenic gene profile in the cells. It is possible that these compounds can induce xenobiotic metabolising activity in these cells.

Work continued on establishing the most relevant cytomic assays to complement the gene regulation and metabonomic changes associated with the carcinogenic profile of the compounds. This work indicated that the assays of most promise include glucose/lactate, cell cycle analysis and epithelial cilia.

A very significant amount of work was carried out in both the human RPTEC/TERT1 and rat NRK-52E cells with 15 compounds including 5 genotoxic carcinogens, 5 non-genotoxic carcinogens and 5 non-carcinogens. The gene array data and the metabonomic data show great promise in differentiating between the classes of compounds.

Table 2. Overview of carcinoGENOMICS Milestones as achieved during the months M37-48.

Milestone No.	Deliverable name	Delivery date	Completed	Remark
M11.2.9	Dissemination of information through the electronic newsletter, based on input of the partners	M37	Combined with M11.2.10	Due to insignificant results from the partners the Project Board decided to combine this Milesto-nes with M11.2.10
M12.9	Plans & budgets for fourth year prepared and approved	M37	√	
M4.4	Test formats for carci-nogenesis using immor-talised primary human lung cells assessed	M38	√	
M9.1	Selection of promising methods for entering pre-validation exercise according to the list of criteria approved by the Management Board	M38	√	
M12.10	Annual Reporting to the European Commission: Periodic Activity Report, Periodic Management Report, DoW	M38	√	
M2.1.4	Upon examination of data collected in pre-vious experiment, all the partners will decide which of the liver models go on into phase II study	M38	√	
M2.2.6	Initiation of carcinoge-nicity testing with com-pounds on the extended list	M39	√	
M11.2.8	Updating of flyer when new partners come in	M40	√	

M3.5	Decision of most suitable cytomic assays	M40	Postponed to M59	
	The results to date indicate that glucose/lactate; cell cycle analysis & epithelial cell cilia are the most promising; interim report available within the consortium			
M11.1.9	Organising a workshop depending upon final input of WG result reports & recommendations	M42	Postponed to Year 5	Postponed and renamed into: M11.1.10: Organising a workshop and setting up an agenda depending upon final input of WG result reports and recommendations
M2.2.7	Establishment of definitive endoderm derived from feeder-free hESC	M42	√	
M2.3.5	Omics data combined with genotypes for the first 5 donors	M42	Postponed	
M52	Trancriptomic data are all available & statistical analyses are ongoing. Metabolomic samples sent to Imperial College for analysis; so far no experiments have been undertaken. This Milestone renamed 'Omics data combined with epigenotypes for the first 5 donors' & postponed to M52			

M2.3.7	Select classic bioacti-vable carcinogens for testing in HepG2 with 'risky' phenotype. Define time & dose	M42	√	
M5.4	Continuation of up-loading transcriptome data sets onto the central data warehouse at EMBL-EBI	M42	Postponed	
M55	Continuously transcrip-tome data are uploaded onto the central data warehouse at EMBL-EBI. Up to November 2010 this has been done for >2000 RNA samples. Still in pro-gress & postponed to M55			
M6.6	Analytical data genera-ted for further sets of samples from WP2-4	M42	√	
M7.3.7	Detailed characterisati-on of compounds	M42	√	
M9.2	Selection of partici-pating laboratories & validation Management Team	M42	√	
M11.2.10	Dissemination of in-formation through the electronic newsletter, based on input of the partners	M43	√	
M11.2.7	Updating of the list of relevant publications	M44	√	
M2.1.5	Based on the results obtained in phase I study, re-define time & dose of the different compounds in phase II	M44	√	

M3.4	Identification of most suitable system to enhance the xenobiotic metabolising capability	M44	√	
M7.1.6	Final version of the whole ISA infrastructure released, as software suite for local use	M45	√	
M7.1.7	carcinoGENOMICS & other relevant or related datasets of interest, stored in the BioInvestigation Index instance at EBI & related public repositories	M45	√	
M7.2.6	Pathway analysis on selected third dataset from consortium, provided sufficient data are generated in the consortium to justify a 3rd analysis (otherwise only Milestones 7.2.2 & 7.2.4. will stand)	M46	√	
M4.8	Learning set of chemicals tested on the Epithelix model	M48	Stopped	Milestone discontinued
M4.9	Genomic data (task 4.7) analysed	M48	Postponed year 5	Analysis on 15 compounds still in progress
M6.7	Multivariate statistical models indicating metabolic response to toxicant exposure generated	M48	√	

It was ultimately decided to go forward with the human RPTEC/TERT1 cells as the kidney model for the pre-validation phase.

WP4: Lung Models

The 3D lung tissue models derived from primary bronchial epithelial cells of 4 different donors, challenged with the 6 'learning set' compounds, revealed discriminatory toxicogenomic profiles. All 4 donors performed well and gave consistent results. Therefore, exposures to 12 additional compounds were performed using exactly the same conditions. In addition, some previous exposures were repeated. Preliminary data analysis demonstrated the reproducibility of responses and revealed again promising results regarding the discriminatory potential.

Besides the use of primary cells to generate lung tissue cultures, considerable progress was made in using immortalised cells for this purpose. After various quality criteria for the cultures were fulfilled, it was decided to start comparing the basal transcription profiles of primary and immortal cultures as well as the transcriptional responses after exposure to 3 chemical compounds. Finally, the applicability of the lung models for exposure to gases were examined, and subsequently a pilot study using 1,3-butadiene was initiated.

WP9: Optimisation & Pre-Validation

WP9 activities began in Year 4. The overall objectives of WP9 are to 1) further develop each test method by testing additional 15 chemicals, and 2) assess test method transferability and reproducibility by testing three coded chemicals in three laboratories for each test model, using the same agreed SOPs and controlled conditions.

The activities of Year 4 were mainly related to the set up of the study plan for the optimisation phase and the co-ordination by partner 14 of its initial activities. This involved intense communication with the different partners from the same WP, as well as from different WPs. First, the most promising test methods for the liver and for the kidney target organs were selected based on transcriptomics data and other characteristics. The methods using the HepaRG and the RPTEC/TERT1 were identified as the methods of choice for the liver and the kidney, respectively. Subsequently, the laboratories participating to the inter-laboratory evaluation and the respective lead laboratories were selected for each organ model. A MT was set up to supervise that the work is conducted following the agreed conditions. This MT selected the chemicals to be used in the inter-laboratory study with the support of the bioinformaticians. The selected laboratories revised their SOPs under the guidance of partner 14. Moreover, training sessions were organised in order to ensure that all participating laboratories have common understanding of the work to be performed in Year 5 and to harmonise their laboratory procedures. In parallel, WP7 started to identify the statistical approaches to be used in the evaluation of the inter-laboratory results.

WP1 with input from other partners and the project advisors finalised the chemical list to be used in Phase II for the further development and improvement of the prediction models. This list of chemicals and the list of chemicals for the inter-laboratory reproducibility were shared with the University of Maastricht who will be responsible for their coding and distribution.

Challenges & Solutions

Given the fact that the carcinoGENOMICS project has entered its last year, the main challenge is to finalise it in due time. This in particular refers to:

- Finalising interlaboratory comparisons of selected cellular models and predictive toxicogenomics
- Finalising testing an additional 15 model compounds per selected cellular model
- Finalising in-depth data analysis through advanced bioinformatics/ biostatistics, including cross-platform, cross-study meta-analyses
- Preparing a final report covering all endpoints at the transcriptomic and cytomic level that allow discrimination between genotoxic and non-genotoxic carcinogens in the selected liver-, kidney- and lung-based *in vitro* models;
- Based on the ultimate outcome of the work, to define conclusions that can be shared with external stakeholders at the last capacity-building workshop.

The solution is that should set backs occur, the Co-ordinator will request an extension of the project's deadline by 6 months.

Next Steps

For Phase II, e.g., the fifth and last year of the carcinoGENOMICS project, selected human cellular models will be challenged with a second series of test selected carcinogens and non-carcinogens, thus providing more information on the accuracy of generated gene signatures for predicting genotoxicity and carcinogencity *in vivo*. In parallel, first steps of the formal pre-validation process will be completed, in particular focussing on the evaluation of inter-laboratory variability of these assays by using a limited set of compounds. These will be the first toxicogenomics assays *in vitro* to be subjected to pre-validation under the guidance of ECVAM. Unfortunately, given time and resources available, this pre-validation work needs to be of modest proportions, implying that for further validating and establishing these promising models, follow-up work will be still required after the carcinoGENOMICS project will have ended. This also related to informing important stakeholders such as regulatory authorities in the domain of chemical safety. Follow-up work is also related to informing important stakeholders such as regulatory authorities in the domain of chemical safety.

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ChemScreen

Chemical Substance *In Vitro/In Silico* Screening System to Predict Human & Ecotoxicological Effects

Grant agreement no: 244236
Project type: Integrated Project (FP7; Environment theme)
EC contribution: € 3,496,724
Starting date: 1 January 2010
Duration: 48 months
Website: chemscreen.eu

Background & Objectives

The complexity of the system of risk assessment of chemicals has led to huge delays. Because of this, the toxicological properties of most industrial chemicals in common use are largely unknown. New legislation such as REACH aims to end this unacceptable situation by modernising and streamlining chemical risk assessment. However, this approach is unlikely to be successful without incorporating alternative, integrated testing strategies in which chemical characteristics are used to more advantage and where costly and time-consuming animal tests are replaced to a large extent by rapid and cost-effective alternative testing methods using cultured cells. This is particularly needed for reproductive toxicity testing of chemicals. Reproductive toxicity is important to assess effects on both human health and the environment, and uses the most animals in toxicity testing. Unfortunately, there are very few alternative methods. We aim to fill this gap and select suitable tests and place them in a more general, innovative and animal-free testing strategy. For this, we are generating a simple, rapid screening system aiming at widespread implementation within the tight time schedule of the REACH programme. It will be a flexible tool that can be adapted and used for applications beyond the scope of REACH and in the post-REACH period. It will use *in silico* methods for pre-screening chemicals for all relevant toxic effects. When found positive, this will be followed by further *in silico* and *in vitro* tests, most of which are available already. To fill the gap of suitable alternative methods for reproductive toxicity testing we will use a novel high-throughput approach combining *in silico/in vitro* methods. In this approach we will

combine knowledge of critical processes affected by reproductive toxicants with knowledge on the mechanistic basis of such effects. Straight forward data interpretation and decision trees will be developed in which all information on the potential toxicity of a chemical is considered. In this way we will provide a cost-effective means to generate a basic set of data on toxicological properties of chemicals and a decision tool to assess if further testing of chemicals is required or can be waived.

Objectives (illustrated in Figure 1)

1. Establish *in silico* pre-screening methods prioritising *in vitro* toxicity testing (WP1, Co-ordinating Partner, see below)
2. Establish a database and an *in silico* prescreen to identify potential reproductive toxicants (WP2, Partner 2)
3. Establishment of sensitive parameters and a medium-throughput 'minimal essential' *in vitro* assay panel (WP3, Partner 5)
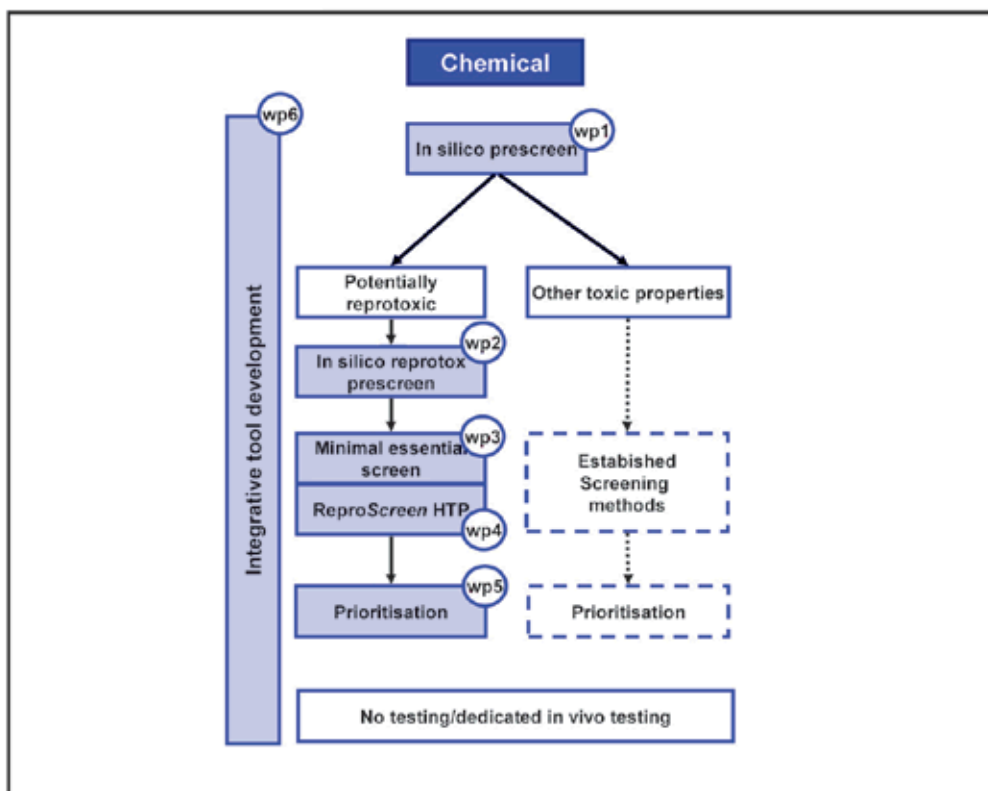

Figure 1. Graphical representation of ChemScreen's major components. The dotted lines indicate areas where alternative methods are relatively well developed or under construction in various (FP) programmes in which partners participate. All workpackages (WPs) are indicated, except WP7 (dissemination) and WP8 (management).

4. Establish a high-throughput mechanistic pathway screen, ReproScreen HTP, for reproductive toxicants (WP4, Partner 8)
5. Integrative methods to predict *in vivo* reprotoxicity for both human- and environmental toxicity allowing informed decisions on eventual further testing (WP5, Partner 3)
6. Integration into one user-friendly tool, including uncertainty assessment (WP6, Partner 7)
7. Efficient dissemination to facilitate widespread implementation (WP7, Co-ordinating Partner)

Deliverables & Milestones Achieved During 2010

The ChemScreen project began on 1 January 2010. The programme aims to collaboratively generate an innovative testing strategy combining unique expertise of the participants. To attain this level of interaction, frequent meetings and workshops have been planned, as well as a high-profile Scientific Advisory Board (Table 1) to help guide this process. In the international advisory board, major stakeholders (JRC/ECVAM, OECD, US EPA, CEFIC, ECETOC) are represented. In 2010, two general project meetings were organised, and one in 2011. These included workshops on topics that are relevant to the scientific programme, including one on chemical selection, one on assay selection and one on integrated testing strategies and *in silico* methods. The project's Executive Board was established and consists of Prof. Dr Albert Piersma (RIVM), Dr. Dinant Kroese (TNO), and Dr. Bart van der Burg (BDS).

Beginning in 2010, the ChemScreen project has entered an important transatlantic collaboration with the US EPA National Center for Computational Toxicology (NCCT), and the Texas Indiana Virtual Star Centre (TIVSC). This has been substantiated with participation of Drs Maria Bondesson (TIVSC) and David Dix (NCCT; chairperson of ChemScreen's Scientific Advisory Board). Agreements on scientific collaboration, data- and chemical sharing have been established.

As one of the project's first deliverables ChemScreen's strategy has been further

Table 1. ChemScreen Scientific Advisory Board.

Supervisory board	Organisation
David Dix (chair)	US EPA / NCCT
Maria Bondesson	Texas-Indiana STAR Centre
Neil Carmichael	ECETOC
Robert Chapin	Pfizer
Mark Cronin	Liverpool John Moores University
Bob Diderich	OECD
Andrew Worth	Joint Research Centre

defined and published in the form of a position paper (Van der Burg et al., 2011).

The work in WP1 started with the establishment and selection of *in silico* pre-screening methods to categorise chemicals, using (quantitative) structure-activity relationships (Q)SARs. This will be done for major classes of toxicity prioritised in REACH (carcinogenesis, mutagenesis and reproductive (CMR) toxicity and persistent, (very) bioaccumulative toxic (PBT/vPvB) compounds). With this we prioritise further *in silico/in vitro* toxicity testing. As a starting point, we are using a unique database which comprises abbreviated predictions from more than 70 (Q)SAR models on endpoints for physico-chemical properties, fate, eco-toxicity, absorption, metabolism and toxicity. A methodology that alerts for potential reproductive toxicity will be developed and integrated all into one module, a fast and efficient toxicity screening tool will be generated based on available *in silico* techniques, mostly QSARs that can identify toxicological profiles that will drive the risk assessment of the chemical, e.g., mutagenic, genotoxic carcinogenic, or endocrine-active properties.

WP2 focussed on expansion of two different databases, which contain information relevant to reproductive toxicity; RepDose (repeated dose) and FeDTex (fertility and developmental toxicity). First reproductive toxicity studies were identified from peer-reviewed publications, as well as other databases provided by other partners. Available studies on reproductive toxicity were selected for data entry. In particular,

the amount of chemicals in FeDTex will be more than doubled in the course of Chem*Screen*, mounting up to over 300. A significant overlap with RepDose is created to allow comparison of *in vivo* data with the same chemical and evaluate possible predictivity of repeated dose toxicity for reproductive toxicity.

WP2 scientists started a unique collaboration with WP3 and the NCCT on the identification of critical endpoints of reproductive toxicity using combined, in depth analysis of databases present at three different locations (i.e., Fraunhofer Institute, RIVM and NCCT). These results will become available in the next period, and will be extended to ecotoxicological endpoints using the EDUKON database present at the University of Konstanz (see WP5).

The aim of WP3 is the design of a 'minimal essential' reproductive toxicity screen, consisting of a number of *in vitro* assays that are representative of those parameters in reproductive toxicity that are crucial for reproductive hazard assessment. A literature survey of past experience will result in a prioritised list of parameters that need to be addressed in hazard assessment. This part of the work is ongoing. Meanwhile, progress was obtained with the optimisation and amendment to higher-throughput of two tests that are very likely to be included in the final battery, namely a genomics-based improved embryonic stem cell test, and steroidogenesis assays. In addition, zebrafish early life stage tests and oocyte maturation/fertilisation tests are being

evaluated for potential incorporation into the assay panel.

WP4 focuses on the establishment of high-throughput screening methods (Repro*Screen* HTP) predictive of reprotoxic potential, based on insight in molecular mechanisms that are relevant to reproductive toxicity. These assays comprise a panel of highly specific CALUX® reporter gene assays, which has been expanded and now includes more than 20 different cell lines, that are run in different assay formats (in particular agonistic- and antagonistic mode), totaling about 40 different assays. The selectivity and the clear mechanistic base of the assays aims at better possibilities for risk assessment and extrapolation to other species in the context of environmental risk assessment.

In addition to these highly selective CALUX HTS reporter gene assays, assays are being established in more complex, differentiated cells such as murine ES cells by introducing reporter systems for signalling pathways controlling key differentiation pathways in embryonic development (so-called ReproGlo assays). In this way, assays for up to 5 signalling pathways that are important for early embryonic development (Wnt/, SHH, TGFβ-, Delta/Notch-, and the RTK-signalling pathway') will be established. All CALUX and ReproGlo assays have now been automated and a series of reference compounds has been used to deselect less informative assays and identify missing endpoints. A further (de)selection of the assay panel will be made based on the critical endpoints identified in WP3,

and additional chemical testing. Data storage and analysis is being set up in collaboration with WP6 partners.

WP5 is establishing integrative methods to predict *in vivo* reprotoxicity for both human and environmental toxicity using *in vitro* benchmark (threshold) concentrations as a starting point. Prediction of the correct *in vivo* dose level at which adverse effects can be expected is one of the key issues. Pharmacokinetic models are being set up describing the bioavailability via relevant routes (oral, dermal, inhalation) and the pharmacokinetics of the most relevant reference chemical classes and generic models to be used for other compounds. In risk assessment, methods determining defined conserved mechanisms of toxicity have a great advantage over more classical 'black-box' approaches, such as morphological endpoints. This is because interpretation of data is much more straightforward. By focusing on molecular mechanisms conserved in various species (e.g., various receptor-mediated processes) possibilities are explored to predict not only for humans but also for aquatic organisms (particularly fish) and thus for ecotoxicological effects. For this extrapolation, methods are being designed to establish if there are conserved molecular mechanisms in aquatic organisms responsive to the action of different chemicals. As one of the first steps, an expansion and restructuring of the large EDUKON ecotoxicological database has been carried out as well as an inventory of possible chemo-biological interactions that can be related to distinct molecular mechanisms.

WP6 is devoted to the setup of a software tool that integrates the methods, modules and databases generated in WPs 1 through 5. It aims to be a flexible and open tool that can be adapted and used for applications beyond the scope of REACH and in the post-REACH period. The tool will take into account the existing landscape of related IT tools (IUCLID5, OECD Toolbox, OSIRIS Web tool, etc.), and will interface with them as required. Concept schemes and the basic design of the software tool have been established at SIM and PGEN. Data integration and analysis methods are being developed using a Bayesian Network (BN) approach using data from various sources including that of the first Chem*Screen* HTP screens and data from NCCT collaborations.

For the dissemination of the project's results regular channels are used, including publications, folders, news items (learn more at *chemscreen.eu*), participation to and organisation of meetings with scientists and various stakeholders, etc. In addition, ample attention is paid to validation and regulatory acceptance of the test methods developed in Chem*Screen*. To this end close contacts with relevant ECVAM and OECD working groups, scientists and regulators are being established. Novel science- and performance criteria-based methods will be employed to validate the Repro*Screen* system, and allow rapid acceptance. Methods aim to be robust and simple allowing widespread dissemination using ample expertise available at Partner BDS to disseminate bioassays worldwide. Methods to screen for endocrine disrupting

compounds, such as the ER- and AR CALUX assay already were pre-validated in the context of the ReProTect FP6 project, and subsequently have been submitted to ECVAM to allow formal validation. Since our battery of tests of reporter gene assays is very comparable to these tests, they may be used as 'validation anchors' in a screening battery. Through close collaboration, synergy will be attained with ongoing framework projects, and the NCCT molecular screening programmes. In order to pre-validate our screening battery and select relevant tests a feasibility study has been initiated early in the project using a set of reproductive toxicants that are run in all available tests.

Challenges & Solutions

The major challenge for the Chem*Screen* project is a timely delivery of an *in vitro/in silico* tool, finding a proper balance between rigorous validation and regulatory acceptance, speed and costs, and coverage of major endpoints relevant for reproductive toxicity. This can never be attained by a single party in such a relatively short timeframe as needed for REACH and related chemical screening programmes. Therefore, the current strong consortium with highly complementary expertise has been built. To be successful, however, it is pertinent that all available technical solutions be considered, and therefore the Chem*Screen* programme actively collaborates with leading groups in the area of rapid screening methods for reproductive toxicity (i.e., the NCCT, TIVSC, JRC systems toxicology unit,

etc.). In addition, to shorten the typically lengthy procedures of regulatory acceptance, interactions with stakeholders and regulators, as well as consideration of alternative methods for validation, are being pursued from the start of the project using methods that have already been set up and pre-validated in the FP6 project ReProTect (i.e., ReproGlo and CALUX methods).

Next Steps

The Chem*Screen* project has made considerable progress at the level of the individual work packages and several integrative projects have already been started between them. Next steps will be focused on HTS data generation and integration to allow selection of the most relevant tests. In this context the identification of critical endpoints of reproductive toxicity is expected to be important. In the next phase of the project intense interactions between work packages, as well as an outreach towards stakeholders, is essential to come to a rapid integrated screening system.

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COMICS
Comet Assay and Cell Array for Fast & Efficient Genotoxicity Testing

Contract number: LSHB-CT-2006-037575
Project type: Specific Targeted Research Project (FP6)
EC contribution: € 3,189,385
Starting date: 1 January 2007
Duration: 36 months
Website: comics.vitamib.com

Objectives

The overall aim of the COMICS project was to develop improved methods for testing chemicals for potential genotoxic and cytotoxic effects; thus to help to limit the amount of animal experimentation by providing validated, reliable *in vitro* assays. Developments were based on the comet assay for DNA damage (single cell gel electrophoresis), the 'cell array' cytotoxicity testing system, selected metabolically active cell lines, and existing assays for DNA repair. Our specific objectives were:

- To increase the throughput of the Comet assay up to 20-fold, using multi-well format and 'cell arrays'
- To develop further the cell array system as a parallel assay for cytotoxicity
- To seek optimal cell types for use in genotoxicity and cytotoxicity testing (e.g., metabolically active HepaRG cells)
- To increase the speed of scoring of comets, by developing an alternative method based on differential fluorescence of DNA in heads and tails of comets, so that scoring is no longer a serious bottleneck
- To use lesion-specific enzymes and inhibitors to measure different kinds of DNA damage – oxidised and alkylated bases, UV-induced damage and bulky adducts
- To develop and compare methods for measuring DNA repair activity
- To develop an approach that combines fluorescent in situ hybridisation with the Comet assay, allowing measurement of gene-specific DNA damage and repair
- To validate the Comet assay in its various forms, assessing reproducibility and

robustness, comparing results obtained with the same test system and the same chemical damaging agents in different laboratories

- To develop reference and internal standards for use in the Comet assay
- To make the various innovative products available for use by companies and researchers investigating DNA damage and repair.

Achievements

- High-throughput Comet assay variants: 12-gel slide format, 48- and 96-gel arrays on GelBond film, cell arrays (individual cells on micropatterned surface of glass plate) all successfully tested as comet assay formats
- Increased speed and efficiency of scoring, based on the Imstar Pathfinder instrumentation and dedicated Comet programme
- Use of lesion-specific enzymes to increase sensitivity and sensitivity of Comet assay
- DNA repair assays based on Comet assay, and on 'Biochips' with oligonucleotide or plasmid substrates
- Detection of gene-specific DNA damage and repair using specific probes
- Calibration of the assay with X-rays (inter-laboratory trial)
- Reference and internal Comet assay standards—based on use of cells with low DNA content (fish erythrocytes) that can be distinguished from sample cells in the same gel
- Validation of the Comet assay variants—comparing with conventional assay, comparing different scoring methods, and testing ability of novel methods to detect genotoxic effects in cell culture.

Continuation of the COMICS Project

Informal links between partners have been maintained, and in several cases there is existing or planned collaboration. Here we describe examples of projects that directly stem from the work done in the COMICS project.

Strand breaks (lysis only)   **Damaged bases (FPG-sites)**
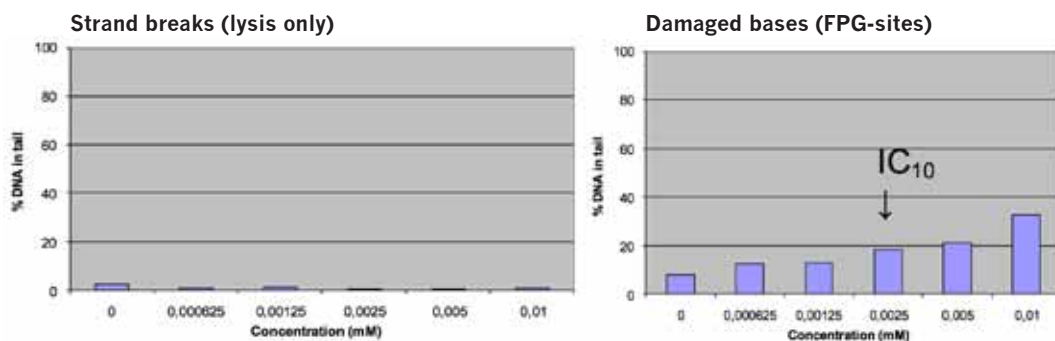

Figure 1. Methylnitrosourea (MNU) treatment of TK6 cells for 3 h. Cells were embedded in agarose and lysed as usual. Incubation with FPG (right hand panel) resulted in a dose-dependent increase in DNA breaks. The increase was significant at non-cytotoxic concentrations (IC_{10} indicates an inhibition of cell growth of only 10%). Unpublished results, Amaya Azqueta and Andrew R. Collins.

Use of Lesion-Secific Endonucleases to Increase Sensitivity of Comet Assay in Genotoxicity Testing

It was clear from the validation studies that we carried out in COMICS that the comet assay in its basic form, without enzymes, is not good at recognising genotoxic chemicals unless they induce strand breaks. This is obviously what would be expected, and yet the basic Comet assay is employed as a test in many commercial laboratories in preliminary screening for DNA-damaging agents. Following up preliminary experiments under COMICS, we at UiO (in collaboration with the University of Navarra) have begun a systematic investigation of known genotoxic chemicals, negative control compounds, and cytotoxic but non-genotoxic compounds. Figure 1 shows, as an example, the ability of FPG to reveal the effect of MNU at very low concentration. So far, we have not found any 'false positives', i.e., non-genotoxic compounds giving an increase in FPG-sensitive sites.

Development of Improved Electrophoresis Equipment (Tanks & Power Supplies) & Use of Robot

Research to optimise Comet assay electrophoresis is continuing (Norwegian Institute of Public Health/Thistle Scientific, in collaboration with a commercial supplier of integrated electrophoresis systems). Technical and theoretical aspects of electrophoresis are being studied. Based on this and on results from the calibration trial within COMICS, improved protocols are being developed. A standard pipetting robot has been purchased by NIPH and is currently adapted for precise gel sample application, in order to facilitate automated scoring of comets.

Publications 2010-11

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Commercialisation

This was an important aspect of the COMICS project, and university/research institute partners worked together with SMEs to develop equipment, reagents, software, stains, etc. for incorporation in the new high-throughput methods. Development of several innovations from the COMICS project is continuing, e.g., a modified electrophoresis tank to take 4 GelBond films (Thistle Scientific); slides coated with a polyacrylamide/ agarose mixture to improve attachment of gels (Severn Biotech); DNA repair biochips (CEA); fish erythrocyte-based internal standards (NIPH). The 12-gel slide chamber is produced on a small-scale (Severn Biotech) and has been very well received by users; there is certainly a potential market for this and other COMICS-designed equipment.

Patents, etc.

- Severn Biotech: UK Patent applied for 12-gel apparatus. Certificate of Design Registration 4018593 for 12-gel Apparatus.
- Trade Mark for PAGAROSE (coated slides). PAGAROSE Patent will be applied for.
- The SMART cube is being trademarked and the Patent application is being written.

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ESNATS

Embryonic Stem Cell-Based Novel Alternative Testing Strategies

Contract number: HEALTH-F5-2008-201619
Project type: Integrated Project (FP7)
EC Contribution: € 11,895,577
Starting date: 1 April 2008
Duration: 60 months
Website: esnats.eu

Background & Objectives

Current toxicity testing in the drug development process is characterised by a number of shortcomings:

- A major part of safety testing takes place late in the research and development cycle, implying protracted experimentation involving high numbers of animals and generating significant costs
- Some *in vitro* assays rely on cell lines of malignant origin or primary cells that are hard to standardise and limited in terms of quantity, homogeneity and genetic diversity
- Existing assay systems based on primary animal and human cell lines do not reliably represent the physiological situation of cells in native tissue.

To overcome these shortcomings, the ESNATS consortium is developing a novel testing system taking advantage of the unique properties of embryonic stem cells (ES cells), including:

- Their characteristic property to self-renew, constituting a potentially unlimited source of cells
- Their pluripotency (i.e., their potential to give rise to all organ-specific cell types), providing a source for cells of different phenotypes required for toxicity testing
- The physiological relevance of ES cell-derived somatic cells for toxicity endpoints, offering a perspective of toxicological in vitro tests with improved predictivity
- Their easy genetic manipulation, allowing use of reporter gene expression as a

powerful toxicity testing tool.

The overall aim of the ESNATS project is to develop a novel toxicity test platform based on ES cells, in particular human ES cells (hES cells), to streamline the drug development R&D process and evaluation of drug toxicity in clinical studies, reduce related costs and thus, to increase the safety of patients while reducing the numbers of test animals due to earlier detection of adverse effects.

To achieve the project goals, a battery of toxicity tests is developed using hES cells subjected to standardised culture and differentiation protocols. By using hES cells, both the effects of test substances on the development of organotypic cells from hES cells and on the differentiated organotypic cells can be studied. State-of-the-art genomics approaches are used to identify predictive toxicoproteomic and toxicogenomic signatures in the *in vitro* cellular model systems developed by the consortium. Dose-response curves obtained from the various *in vitro* systems will be translated into critical dosage levels *in vivo* by using toxicokinetic modelling approaches.

In the final two years of the project, the individual assays will be integrated into an 'all-in-one' testing strategy using selected hES cell lines to answer various toxicological questions. Such a strategy will avoid having to establish several *in vitro* tests based on cells of various origins such as primary cells, cancer cells, etc.

This approach will be supported by developing concepts for automated ES cell culture, providing the basis for scale-up of ES cell-based *in vitro* testing.

In the final stage of the project, successfully developed tests will be combined in a testing strategy and a proof-of-concept study will be performed.

Deliverables & Milestones Achieved During 2010

See Tables 1 and 2.

Significant Results

Set-up of the Strategy Working Group and Definition of a Test Strategy

In the third year of the project, a specific task force, the Strategy Working Group, was set up to define the overall test strategy to be applied in the two last years of ESNATS. Further to the recommendations of the Strategy Working Group, it was decided to focus on the topic 'prenatal toxicity with emphasis on the nervous system' examined in hES cells and cell types derived thereof. Spermatogenesis (murine model) is therefore no longer part of the ESNATS objectives. Table 3 presents the test systems corresponding to the focus of the ESNATS test strategy.

It was further decided to include in this test strategy a 'biomarker identification study' to be performed with the most advanced test systems. The aim of this study is to identify gene expression signatures by gene array analysis and to establish

Table 1. List of deliverables due in 2010 and summary of progress.

Del n°	Deliverable Name	Summary of Progress
D0.1.3	Ethical workshops on assessment and guidelines for the use of human ES cells for *in vitro* toxicology (1st of 3)	Internal workshops have been organised during the ESNATS summer schools/ General Assembly meetings; a public workshop will be organised within the ESNATS final workshop in Jan/Feb 2013
D0.1.2	Ethical assessment & guidelines for the use of human ES cells for *in vitro* toxicology	A first draft was provided but the final version has not yet been published; it will be published in 2011
D0.3.1	Approaches to standardising culture protocols	Manual procedures for expanding undifferentiated hES cells currently in practice or developed in the context of other ESNATS work packages were made, for purpose of making recommendations on how to enable the procedures to be automated & scaled-up
D1.1.2	In vitro screening tests using drug candidates in the gametogenesis system	First *in vitro* tests have been carried out; however, due to the termination of the partner doing this work in the project as well as the change of focus in the ESNATS strategy, this work will not be pursued
D1.3.2	Definition of hES cell trophoblast model structure	The hES cell derived trophoblast model was structurally & functionally characterised for purposes of serving as an early embryo & trophoblast toxicity model
D1.4.3	Standardised protocol for hES cell cardiac differentiation	SOP for cardiac differentiation of H9 ES cells was established
D1.4.6a	Database of raw data & interpretation of results of the teratogens tested (hES cell neuronal teratogenicity *in vitro* test)	Initial results generated by the training set of 8 compounds using the *in vitro* model for human early embryonic neural development were summarised in this deliverable
D1.6.5	Collection of chosen, suitable biomarkers to monitor hES cell differentiation	Protocols for the spontaneous multilineage differentiation of hES cell derived microtissues in 96-well plates were developed
D1.7.1b	Periodic SP progress report (part of ESNATS periodic progress report)	A summary of the progress in SP1 was provided

D2.1.10	SOP for human mixed cultures	The standard operation procedure (SOP) for derivation of mixed neural cultures from neural precursors is described in this deliverable
D2.1.7	SOP for hNPC	This standard operation procedure describes the differentiation into neural rosettes within neural spheres derived from hES cell lines HES1 & H9
D2.1.8	SOP on measurement of neurite length	This SOP describes a method to measure overall neurite length in form of measurement of neurite mass
D2.1.9	Transgenic mES cell lines	Several genetically modified mouse ES cell lines were generated, with the goal to develop readouts for neurotoxicology testing that are available for ESNATS members
D2.2.3	Report on effects of tool compound set on differentiation of engineered mESC (developmental neurotoxicity)	It was investigated whether genetically engineered mES cell lines were useful tools for detection of effects of tool compounds
D2.3.2	Test of metabolism-requiring compounds on mES cell-derived cultures	It was evaluated whether the neuronal system developed and described in deliverable D2.2.1 ('Compilation of a protocol & tool compound list for pre-validation of mature ESC-derived neurons as in vitro alternative method for toxicity testing') is capable of distinguishing between non-toxic compounds & their toxic metabolites
D2.4.1b	Periodic SP progress report (part of ESNATS periodic progress report)	A summary of the progress in SP2 was provided
D3.2.1	First developmental toxicity signatures	The first developmental toxicity signatures for the two developmental toxicants cytarabine & thalidomide using hES cells were identified

D3.3.1	Toxicity gene expression signatures of reference compounds for murine ES cell-derived neuronal cells, spermatocytes and primary hepatocytes	The first toxicogenomics expression signatures of reference compounds for murine ES cell-derived neuronal cells & spermatocytes were generated & first conclusions derived from these experiments
D3.4.1	Central core data repository & data extraction procedures	The core data repository was set up with the management of transcriptomics & proteomics experimental data
D3.6.1b	Periodic SP progress report (part of ESNATS periodic progress report)	A summary of the progress in SP3 was provided
D4.1.1	Metabolic activities of mouse hepatocytes in transwell culture	It was analysed whether hepatocyte sandwich cultures show a time-dependent formation of metabolites over an incubation period of 24h using diagnostic substrates
D4.2.1	Toxicity and genotoxicity data of the test substances (all data with & without metabolic activation)	An in vitro system with primary hepatocytes cultivated in collagen sandwiches that can serve as a metabolising system for neurotoxicity testing, has been established
D4.3.2	Integrated approach of in vitro testing including metabolising system and PBPK modelling to be used to predict in vivo effect levels for reproduction toxicity & CNS toxicity	A two-step in silico approach to extrapolate in vitro toxicity data has been established, determined in in vitro assays for reproduction & neurotoxicity by other ESNATS partners in SP1 & SP2, to in vivo effect levels for these two toxic endpoints.
D4.4.2	Report on expression profiling and metabolic activities of mES cell derived hepatocyte-like cells	Mouse ES cells were used to express a reporter/selection vector with the eGFP gene & a puromycin resistance (Pac) cassette driven by a common a-fetoprotein (AFP) gene promoter
D4.5.1b	Periodic SP progress report (part of ESNATS periodic progress report)	A summary of the progress in SP4 was provided
D0.6.3	Periodic Report for period 2 (M13-M24)	The periodic report for the period 2 was submitted to the EC

D0.3.2	Cell culture automation: concepts	Requirements for an automated ES cell maintenance system were provided, based on the needs expressed in D0.3.1 & the results of numerous interviews with both ESNATS consortium members & other research institutions
D1.4.4	Report on the most suitable hES cell lines for establishment of neuronal teratogenicity *in vitro* tests	Data obtained with different ES cell lines in the *in vitro* neural teratogenicity test was compared, in particular cytotoxic & lineage-specific toxic effects of different compounds during the neural differentiation process of HUES1 & H9 cell lines
D1.4.5	Report on the characterisation of hES cell derived cardiac cells & predictive endpoints	Results of the differentiation of hES cells into cells corresponding to the cardiac compartment were described
D0.4.5b	Summer school	The second ESNATS summer school was held in Tallinn, Estonia on 19-23 Sept. 2011
D0.5.6	Draft plan for using & disseminating knowledge	The draft plan for disseminating knowledge was updated with the plan for using knowledge
D1.6.6	Report on the universal applicability of the *in vitro* development model as determined by comparative testing of several Cellartis hES cell lines	Several different Cellartis hES cell lines, developed either in a feeder-based or in a feeder-free system, were evaluated & compared; cells either seeded as clusters or as single cells with the possible survival effect of a 'survival compound' were evaluated
D2.1.11	Optimised SOP for mini-brains (modification after electrophysiological characterisation)	The electrophysiological characteristics of spontaneous & evoked field potentials from neural networks derived from mouse ES cells were described
D1.2.2	Expression profiles of hES cells after exposure to reference compounds & identification of pertinent toxicity biomarkers at the transcriptional level	Pluripotent hES cells were treated with toxicants at sublethal concentrations (IC10) & the expression pattern of toxicity biomarkers after exposure to reference toxicants was analysed by semi quantitative RT-PCR & RT-qPCR

Table 2. List of milestones due in 2010 and their status.

Mile-stone nº	Milestone name	Status
4	Tests for neuronal teratogenicity	Was attained with the delivery of D1.4.6b Database of raw data & interpretation of test results
5	Developmental neurotoxicity on mES cells	Was attained with the delivery of D2.2.3 Report on effects of tool compound set on differentiation of engineered mES cells (developmental toxicity)
6	Standardised cell production & automation concepts	Was attained with the delivery of D0.3.2 Cell culture automation – Concepts
7	hES cell assay of early human development	Was attained with the delivery of SOPs for neural & cardiac differentiation
8	Expression profiles of hES cells after exposure to reference compounds	Was attained with the delivery of D1.2.2 Expression profiles of hES cells after exposure to reference compounds & identification of pertinent toxicity biomarkers at the transcriptional level
9	Standardised cell lines, protocols for CNS toxicity	Was attained with the delivery of SOPs (D2.1.4, D2.1.6, D2.1.11)

an algorithm that allows identification of compounds that act by a certain toxic mechanism or induce a specific phenotype in a pathway-based approach.

Further Development & Implementation of the ESNATS Test Strategy

The further development and implementation of the suggested test strategy was then elaborated in a so-called 'roadmap', where the detailed planning of each step of the test strategy is described. As illustrated in Figure 1, a test battery is developed to assess different aspects of prenatal toxicity, such as functional impairments and changes in the differentiation capacity after exposure to well selected reference compounds. On top of this, a biomarker identification study is being carried out to identify gene expression signatures by gene array analysis and to establish an algorithm that allows identification of compounds that act by a certain toxic mechanism or induce a specific phenotype. The test battery and the gene array chip will then be challenged with compounds under blinded conditions and the predictivity of the tests will be assessed. Biostatistics will be used to evaluate specificity, sensitivity and predictive capacity. PBPK modelling will allow the extrapolation of *in vitro* data to the *in vivo* situation.

Experimental design for toxicity tests participating in the battery approach is based on the following:

- Definition of the test method including its biological basis (test system) and a rationale for the relevance of the results produced such as the endpoints to be measured and a rationale or decision criteria for how the results are to be interpreted
- Definition of the toxicity range of test compounds in the test system
- Definition of basic characteristics of the test system and test method: dynamic range of the endpoint, detection limit, stability of the readout
- Data on response characteristics of the endpoint

- Data quality and statistical evaluation.

For the gene array study, the following experimental design has been agreed:

- A '2+1 study', i.e., using two positive and one negative compounds, should enable an initial assessment whether the cell systems deliver reproducible and reasonable expression data. As positive control, methylmercury will be tested by all test developers participating in this study, as well as one compound to be freely chosen by the test developer based on the relevance of the compound for the test system. As negative control, D-mannitol will be used by all test developers.
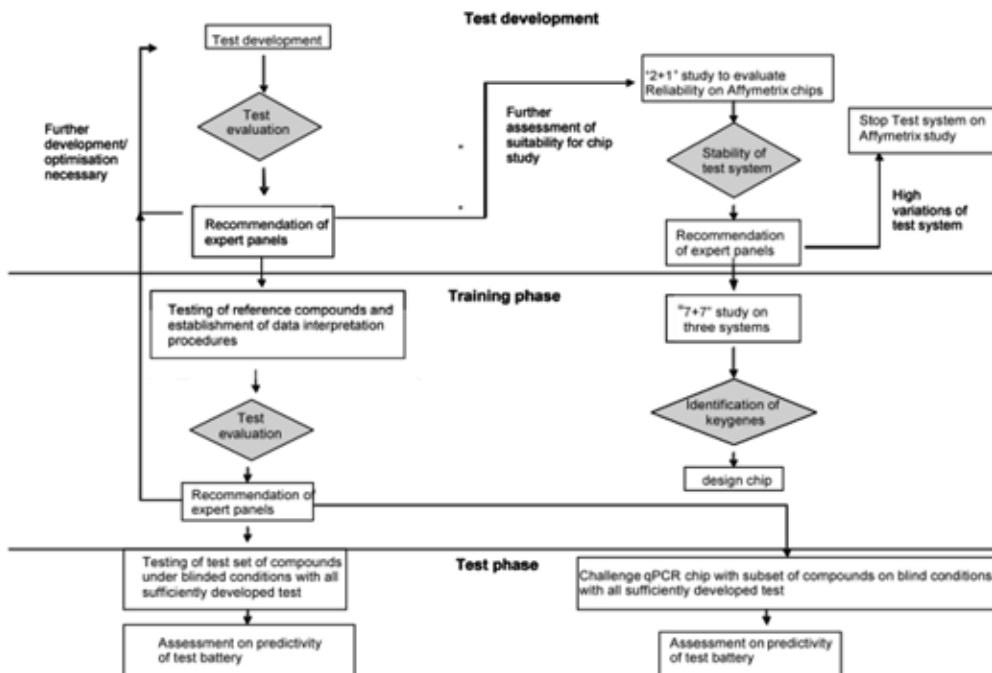- Once the data from the '2+1 study'

Figure 1. Overview of ESNATS test strategy.

Table 3. Test systems corresponding to the focus of the ESNATS test strategy.

Partner	Test systems
UKK	- UKK1: Toxicity assessment in human embryonic development using H9 hES cells, feeder-free, critical window, exposure from day 0 or from day 10 - UKK2: Pre-implantation embryotoxicity based on hES cells; un-differentiated ES cells, pluripotency factors, first test the system, qPCR, Affymetrix-array
CELLARTIS	- Developmental toxicity assay using hES cells, feeder-free, early steps of development till germ layer - Presence and absence of bFGF
UEDIN	Preimplantation embryotoxicity based on hES cell trophoblast models
JRC	Toxicity assessment in human embryonic early neurogenesis/ neural development using H9 hES cells
Avantea	Neural teratogenicity HUES1 line
CellCure	- hES cell-derived dopaminergic neurons - Assaying dopaminergic neurons for developmental toxicity - Assaying dopaminergic neurons for acute toxicity
UKN	Early and late developmental neurotoxicity of CNS & PNS cells: - UKN1: hESC –developmental toxicity during the generation of neuroectodermal cells (NEC) - UKN2: hESC –differentiation of neural crest stem cells (NCSC) to test toxicity to the developing peripheral nervous system
UNIGE	Neurotoxicity, two dimensional & 3-dimensional neural cultures: - UNIGE1: Human mature neurons (2D neurite extension, FACS analysis, regular tox tests; 3D histology, protein expression) - UNIGE 2: Human mature neurons 3D electrophysiology, +/- stimulation

are confirmed, a '7+7 study' will begin with those test systems that have delivered promising results in the '2+1 study'. In the '7+7 study', seven positive and seven negative compounds that have been reported to be neurotoxic in vivo and to inhibit neurite outgrowth in vitro will be tested to identify a specific mode of action or inducing a specific phenotype.

- All experiments will be carried out in five biological replicates.
- Three concentrations will be tested: 1) solvent control, 2) IC10 and 3) IC10 x 0.25
- Biomarkers identified in the different tests will then be assembled

in a multiplex chip, which will be challenged with compounds under blinded conditions as part of the overall testing strategy.

Assessment of ESNATS Test Systems

To assess the readiness of the ESNATS test systems, an evaluation was carried out during the period, both by the Steering Committee and by a specific Evaluation Group, composed of representatives of the project. The following evaluation criteria were applied:

- Availability of SOP
- Reliability of the test
- Acceptance criteria
- Negative and positive controls
- Non-specific controls (depending on system)
- Biological relevance of the test system

The conclusions from the two expert panels were that although significant results have been obtained, most of the test systems would benefit from further optimisation in order to be included in the overall test battery. On the other hand, three test systems were considered ready to start the gene array study: UKN1, UKK1 and JRC. These test systems have been submitted to the '2+1 study'; others might be included depending on results obtained. A new evaluation of test systems to be included in the test battery will be made in month 45.

Challenges & Solutions

The risk in such a project could be that a lot of interesting components (i.e., cell models, protocols, etc.) and data are produced, but the step of assembling the components to produce a range of reliable test systems, for a specific purpose, and to carry out the actual analysis of results, is left too late. To address this risk, the ESNATS partners elaborated a roadmap describing the detailed 'retroplanning' for implementation of the ESNATS test strategy within the project duration.

Next Steps

As mentioned earlier, three reliable test systems have been selected for an initial 'gene array study'. In Phase 2 (training phase) of the project, 3-4 robust test systems covering different critical time windows of neuronal cell differentiation are trained with prenatal toxicants leading to the identification of a panel of marker genes covering a wider range of prenatal toxicity. Then, 7 positive and 7 negative compounds covering various toxicological mechanisms relevant for each particular time point defined by the test developers individually according to the suitability for the respective systems will be used in order to identify a range of potential marker genes. Meanwhile, further development of tests will take place in order to fulfill recommendations of the expert panels and thus, to be ready for the participation in Phase 3.

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INVITROHEART
Reducing Animal Experimentation in Drug Testing by Human Cardiomyocyte *In Vitro* Models Derived from Embryonic Stem Cells

Contract number:	LSHB-CT-2006-037636
Project type:	Specific Targeted Research Project (FP6)
EC Contribution:	€ 2,701,611
Start date:	1 January 2007
Duration:	36 months

Website: er-projects.gf.liu.se/~invitroheart

Objectives

The objective of INVITROHEART was to establish stable cell lines that reliably reflect human cardiomyocyte properties by the development of an *in vitro* model that is derived from human embryonic stem cells (hESC). The aim was to deliver a trustworthy and regulatory acceptable *in vitro* model that can be used by pharmaceutical and non-pharmaceutical companies to replace experimental animals in 1) investigations on pharmacological toxicity and safety of compounds in the drug discovery and development processes, and 2) the testing of toxic effects of chemicals according to the new Registration, Evaluation and Authorisation of Chemicals (REACH) regulation. In the pharmaceutical industry, reliable *in vitro* cell models would contribute to replace current techniques with animal experimentation in the selection and optimisation of lead compounds and in documentation of a selected drug candidate before it enters clinical phases. In toxicity testing of chemical substances replacement of animal testing methods can also be realized in the cosmetics, food, and commodity chemicals industries.

Main Results

The results of the INVITROHEART project are reviewed in *Journal of Applied Toxicology*[1]. Full reporting of project results is available in the Publications listed below.

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1   Mandenius CF, Steel D, Noor F, et al. Cardiotoxicity testing using pluripotent stem cell-derived human cardiomyocytes and state-of-the-art bioanalytics: a review. J Appl Toxicol. 2011; 31, 191-205.

The most significant achievements of INVITROHEART have been:

- Protocols for generation of cells with characteristics of human cardiomyocytes from hESC have been established and sufficient number of cells have been produced for thorough characterisation of these cells
- The hESC-CM (Figure 1) have been characterised and the cells express high levels of ion channels and other cardiac markers demonstrating successful differentiation into a cardiomyocyte phenotype
- The hESC-CM have been compared with adult human cardiac tissue and the levels of several cardiac markers are of similar or higher magnitude as compared with the adult tissue
- Functional characterisation of the hESC-CM has been achieved with electrophysiological assessment using MEA technology and cells have been demonstrated to respond to established pharmaceutical blockers of specific ion channels with expected effects
- Development of a prototype electrophysiology sensing technology, including development and optimisation of appropriate software, enabling recording of hESC-CM action field potentials
- Development and optimisation of a prototype optical microsensor technology for oxygen consumption measurements (respiration) and generation of data demonstrating that this technology is suitable for toxicity testing of pharmaceuticals

- Several cell-based assays with different toxicological endpoints have been thoroughly evaluated. Extensive data generation with test compounds and optimisation of the calculation of toxic effects have been achieved. All partners use test compounds from a defined list and from the same provider
- Several assays have been generated to evaluate toxicological effect of hESC-CM on a single-cell basis
- Test systems for detection of cellular effects at subtoxic concentrations of test compound were developed based on metabolomics and fluxomics modelling
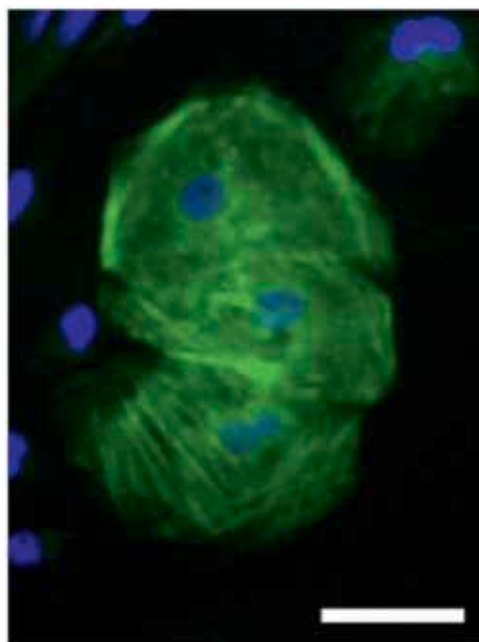

Figure 1. Dissociated hESC-CM stained for the cardiac specific protein troponin, indicating that the phenotype is retained when cardiomyocytes are in a single cell format (troponin, green; nuclei, blue; scale bar, 50 µm). This shows potential for use in single cells applications, such as voltage clamp analysis and immunohistochemistry.
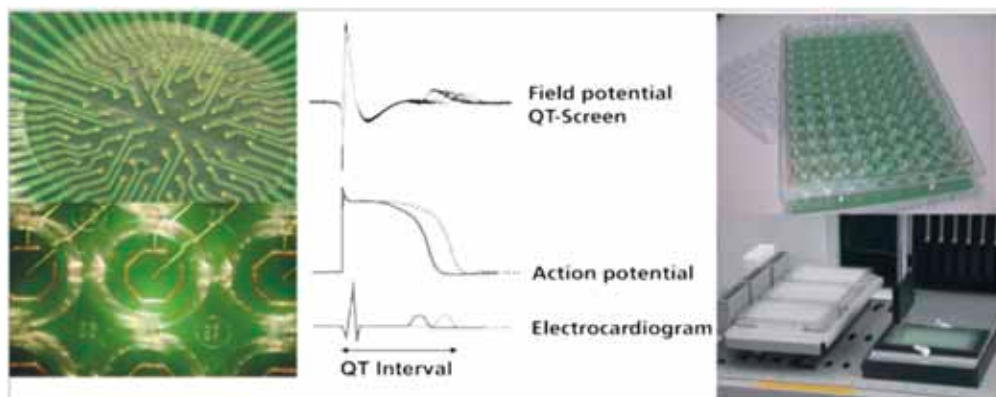
Figure 2. In the left panel the transition from the 60-electrode ecoMEA to a single electrode in a well of a 96-well plate is shown. The middle panel aligns an actual QT-Screen recorded field potential with illustrations of corresponding action potential and electrocardiogram measurements under control conditions and with prolonged QT-interval.

- Detection methodology for cardiac biomarkers has been optimised for surface plasmon resonance technology with hESC derived cardiomyocytes.

In conclusion, these results demonstrate the potential to establish a testing platform for cardiotoxic effects by chemicals and pharmaceuticals—the main objective of the INVITROHEART project.

Spontaneously beating syncytia of cardiac myocytes differentiated from human embryonic stem cells can by electrophysiological recordings be used to address parameters relevant for safety pharmacology. These recordings can be performed by non-invasive extracellular electrophysiology, such as the micro-electrode arrays (MEAs) and QT-Screens systems (Figure 2). Both of the systems have unique advantages: the MEA system allows a high spatial resolution of electrophysiological mapping, whereas QT-Screen offers an increased throughput by recording from 96 electrodes in parallel. Both systems address cardiac safety pharmacology: The MEA system focuses on QT-Prolongation and proarrhythmic events (re-entry, conduction velocity, early- and delayed after depolarisation) whereas the QT-Screen system focuses solely on the shape of the cardiac field potential (QT-prolongation, sodium channel block, calcium channel block). The goal is to use both systems for screening drugs in early safety studies in the process of drug development. Presently, we also use the system for optimisation of the cell differentiation process. By manipulating the signalling pathways involved in hESC-CM differentiation we have increased the yield of cardiomyocytes substantially as well as reduced the yield variation.

Different protocols for cardiogenic differentiation lead to different phenotypes (sinonodal, atrial, ventricular) and different ages of the cultures represent different development stages. With a set

Figure 3. The Sensor Dish Reader (SDR) system from partner PreSens GmbH. This optical fluorescence-based sensor technology allows for optical on-line respiration measurements and monitoring of pH in the cell cultures. Monitoring of the important physiological parameters pH and oxygen is essential for assessing the physiological condition of a cell culture. Consequently, this also becomes an efficient means for detection of toxic effects on the culture. Optical micro-sensors for pH and oxygen offer many advantages over other sensor systems, such as non-invasive and non-destructive measurement from the outside, through the transparent wall of a small bioreactor or cell culture plate. Optical sensors can also easily be combined with other biosensor methods. The panels show culture plates placed in a cell culture incubator for on-line monitoring; one unit of culture well with fluorescence spot; SDR plate optimised for reduced size wells.

of reference compounds the cells are characterised and ranked for suitability in safety screening.

An aluminum holder for small plastic vessels (from 96-well strip plates) with integrated optical oxygen sensors were tested and evaluated for use with the SensorDish Reader for respiration measurements (Figure 3). Thus, less sample volume is necessary, which significantly reduces the numbers of cells needed for detection of the oxygen consumption. For homogenisation of the oxygen ingress in the 24 wells, a cover clamp system (from Applikon) was modified for use with the SensorDish Reader. The homogeneity of the oxygen ingress into the wells using this system was tested successfully with the sodium sulphite method.

Publications 2010-11

1. Mandenius CF, Steel D, Noor F, et al. Cardiotoxicity testing using pluripotent stem cell-derived human cardiomyocytes and state-of-the-art bioanalytics: a review. J Appl Toxicol. 2011; 31, 191-205.
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Patent Applications / Exploitable Results

Patent application: Cardiomyocyte-like cell clusters derived from hBS cells (US provisional 60/960,160, US provisional 60/996,768 and Danish Patent Application No. 2007 01345).

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LIINTOP

Optimisation of Liver & Intestine *In Vitro* Models for Pharmacokinetics & Pharmacodynamics Studies

Contract number: LSHB-CT-2006-037499
Project type: Specific Targeted Research Project (FP6)
EU Contribution: € 2,933,291
Starting date: 1 January 2007
Ending date: 30 June 2010
Website: liintop.cnr.it

Objectives

The main aim of the project was to provide optimized protocols and experimental *in vitro* models for testing intestinal and liver absorption and metabolism of molecules of pharmacological interest. The scientific and technological objectives of the project can be divided in five main areas, as follows:

1. Characterisation and/or production of advanced *in vitro* liver and intestinal models in order to provide improved performance in screening and testing of new drugs' absorption and metabolism:

    *   Comparison of selected functions in the hepatic and intestinal *in vitro* cellular models with the corresponding normal human tissue *ex vivo* (i.e., primary human hepatocytes or human intestinal epithelium)
    *   Characterisation of available intestinal cell lines and selection of the most appropriate model and of the optimal conditions to obtain good and reproducible differentiation
    *   Optimisation of the culture conditions to make the models stable over time for specialised functions
    *   Testing a new strategy using chromatin-remodelling agents (histone deacetylase inhibitors) to induce differentiation in primary hepatocyte-based models
    *   Setting up new approaches to generate metabolically competent human hepatic cell lines. This includes genetic manipulation of existing cell lines (HepG2, HepaRG) transfected with key transcription factors, in order to allow an appro-

priate expression of the differentiated phenotype
- Optimisation of the culture conditions to differentiate adult bone marrow stem cells into functional hepatocytes
- Development of more complex cell co-culture models to combine absorption and metabolism in intestine and liver.

2. Identification of drug transport and metabolism pathways in the available hepatic and intestinal *in vitro* models:

- Modulation of their expression by culture conditions
- Development of high-throughput methodologies for their study.

3. Determination of cellular and molecular targets as endpoints of drug exposure in intestine and liver with respect to:

- Effects on cell proliferation (e.g., cell cycle control, apoptosis/necrosis)
- Effects on differentiated functions (e.g., protein secretion, cell junctions, expression of genes involved in transport and metabolism).

4. *In silico* approaches to modelling the liver and the intestine:

- Development of mechanism-based pharmacokinetic models
- Exploration of the predictive utility of new *in vitro* models

- Identification of areas requiring refinement for future *in vitro* models.

5. Determination of the transfer potential of the developed *in vitro* models for their utilisation within the industrial setting, that derives from the close collaboration within the project of research academic institutions and SMEs.

Experimental Design & Results Achieved

Partners working with cellular models have mainly dealt with objectives 1 and 2, trying to characterise and optimise the cellular models already available or under development in the areas of hepatocytes and enterocytes. The activities performed and the results achieved are described below.

Hepatic Cell Models

Different models and approaches were explored for the hepatic differentiation, since the major problem with hepatocytes *in vitro* is the rapid loss of their specialised functions.

The first approach (by partners INSERM, BPI, Novomass, and Advancell) has been the extensive characterisation and optimisation of the HepaRG cell line. HepaRG, a human liver cell line of tumour origin, has been extensively characterised for the expression of bio-transformation activities such as several major CYPs, antioxidant activities and efflux transport activities (BSEP and MRP2). Differentiated HepaRG

cells express most of the functions expressed by primary human hepatocytes. A transcriptomic study using pangenomic microarrays showed that they expressed around 80-85% of the genes found in human hepatocytes. Most of the phase 1 and phase 2 drug metabolising enzymes as well as plasma transporters are active in HepaRG cells and responsive to inducers and inhibitors. (Data on phase 2 enzymes and transporters are also related to WP3, which was dealing with metabolism and absorption.) The levels of several functions, including some CYPs such as CYP3A4, are dependent on the presence of DMSO at a concentration of 2% (or close) in the culture medium. HepaRG cells can be seeded at low- or high-density. When they are differentiated they retain their drug metabolism capacity and their responsiveness to prototypical inducers for several weeks at confluence. The levels of functions and fold-inductions after exposure to inducers are dependent on culture conditions (presence or absence of fetal calf serum, DMSO). The results are reproducible from one passage to another (studies carried out between passages 10 and 18).

At the genomic level, comparison between HepaRG and normal human hepatocytes has shown a very good agreement in gene expression and modulation. According to the studies performed in the project, the HepRG cellular model has been confirmed as the best hepatocyte *in vitro* model presently available for pharmaco-toxicology studies. The model was also optimised with respect to maintenance and long-term storing conditions. It was demonstrated that all the most relevant drug metabolism activities are maintained including phase-II, UDP-Glucuronosyltransferases (UGT) and sulfotransferases (SULT).

Taken together all the studies performed on HepaRG cells have shown that these cells are unique and metabolically close to human hepatocytes. Their long-term functional stability at confluence makes them suitable for investigating drug metabolism parameters and both acute and chronic ef-
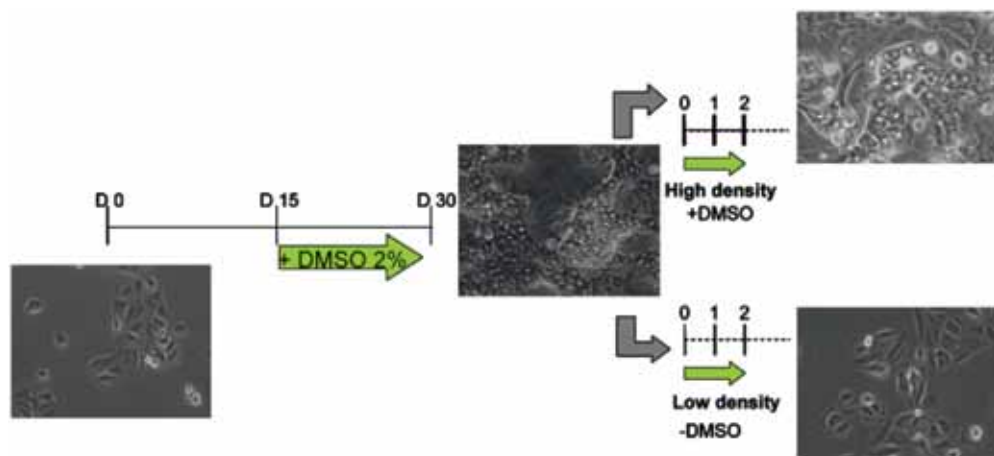

Figure 1. Experimental design for cultivation of HepaRG cells.

Pool 1 22179

Pool 2 22967

781 | 2146 | 593

18674

578 | 1554

4356

25162

HepaRG

74.2 % of genes expressed in HepaRG cells were found in human hepatocytes

84.2 % and 81.3 % of expressed genes in the pool 1 and pool 2 (respectively) of human hepatocytes were found in HepaRG cells

		Number of expressed genes	Number of common expressed genes	
HH	Pool 1	22179	20820	18674
	Pool 2	22967		
HepaRG	P9	28834	25162	
	P10	27122		
	P12	30737		

Each pool of human hepatocytes (HH' was from three donors. HepaRG cells were from 3 diffeaent passages
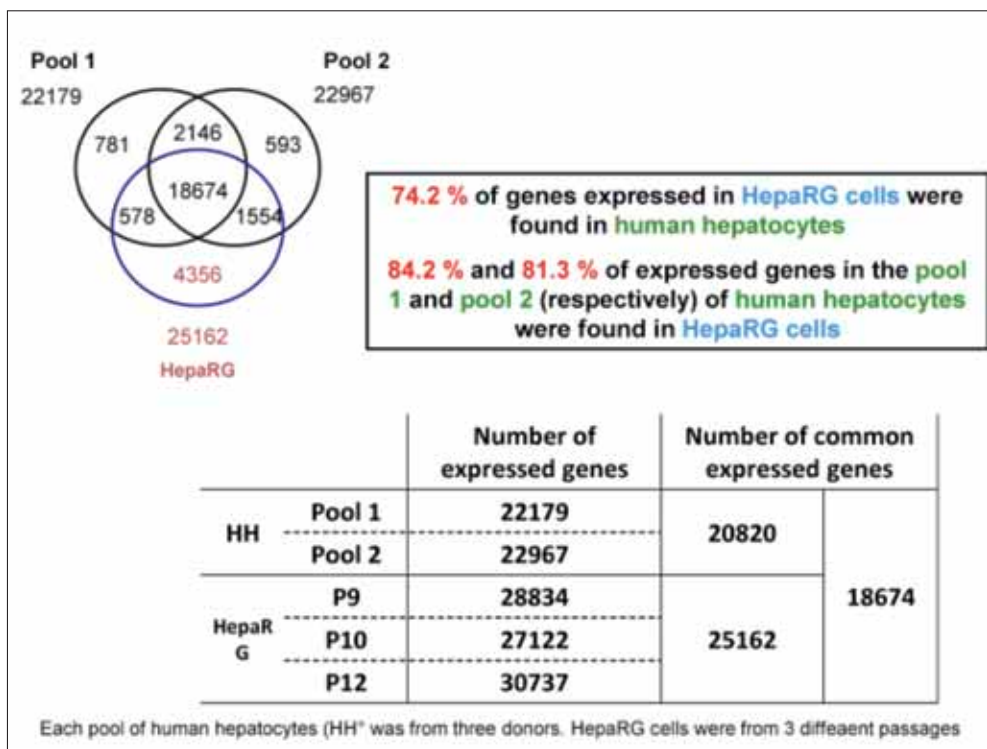
Figure 2. Gene expression in human hepatocytes and HepaRG cells using pangenomic oligonucleotide microarrays.

fects of xenobiotics in the human liver.

A second approach (partner HULAFE) consisted in developing viral vectors to transfect the human hepatic cell line HepG2 with transcription factors, regulating the expression of CYP genes. However, the viral vector employed for transfection has so far only allowed transient expression of CYP activities. Moreover, it became clear that co-transfection of several factors will be needed to achieve functional expression of several metabolic activities. The use of viral vectors for transfection of HepG2 with genes encoding for the transcription factors regulating the main metabolic enzymes (CYPs) has shown that

adenoviruses transfection is working, but with transient activity. In fact, after cell sorting the transfected cells died: this may be attributed rather to the characteristics of hosting cells (i.e., HepG2 cells), than to the genetic manipulation, that proved to be efficient to achieve the envisaged goals in Hela cells. The use of lentiviruses may offer better possibilities for the maintenance of the transfected activities.

The third approach (partner VUB) was the development of phenotypically stable and functional cultures of primary rat hepatocytes by using histone deacetylase (HDAC) inhibitors, e.g., Trichostatin. A novel strategy to differentiate rat and hu-
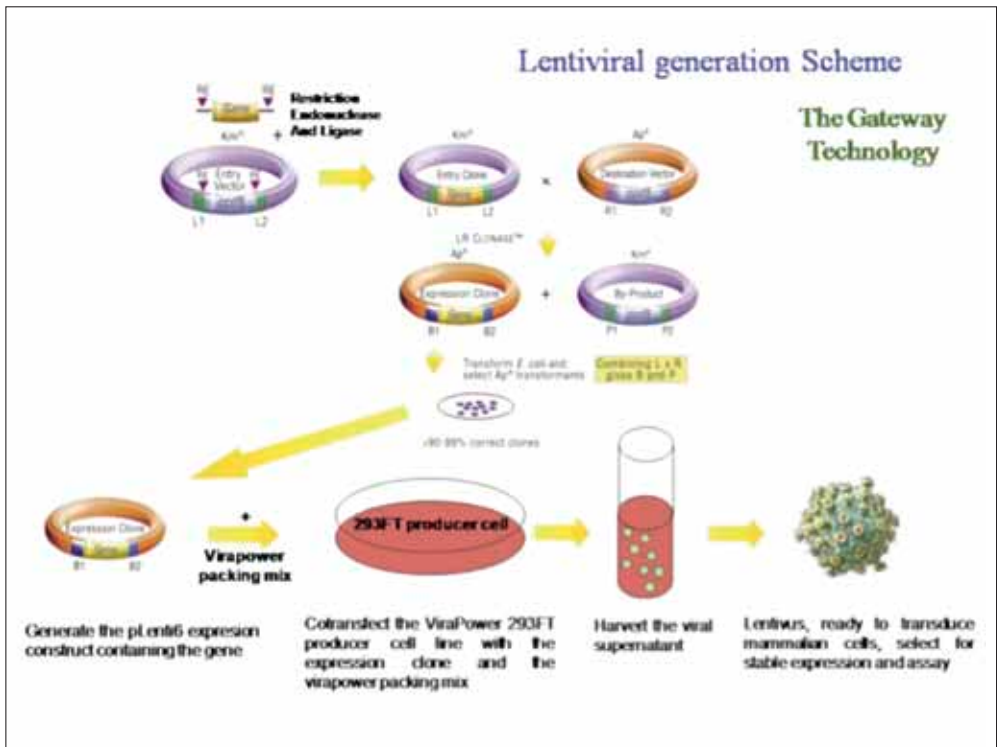
Figure 3. Scheme of lentivirus generation system.

man mesenchymal progenitor cells (MPC) into functional hepatocytes via sequential exposure to hepatogenic factors [fibroblast growth factor (FGF-4), followed by hepatocyte growth factor (HGF), followed by HGF + insulin-transferrin-sodium selenite (ITS) + dexamethasone (Dex)], reflecting the order of secretion during liver embryogenesis *in vivo* was previously developed (Snykers et al., 2006; 2007a). In order to gain information with respect to the general application potential of the established sequential set-up, it was investigated whether primitive cells from another origin than bone marrow, could also be triggered to undergo functional hepatic differentiation, applying a similar sequen-tial strategy as previously optimised for rat and human MPC. More specifically, the sequential-culture strategy by combining gradual and sequential exposure of hepatogenic factors to mimic more accurately the cross-talking signalling network during embryonic liver development *in vivo* was finely tuned. Oncostatin M (OSM) was additionally applied to the last steps of the differentiation protocol in order to complete hepatic maturation. As it was found that pre-treatment with FGF-4 generally induces dedifferentiation of rLEC-derived hepatic cells, cells were immediately exposed to HGF. In addition, it was examined whether TSA, a potent histone deacetylase inhibitor, could expedite the hepatic dif-
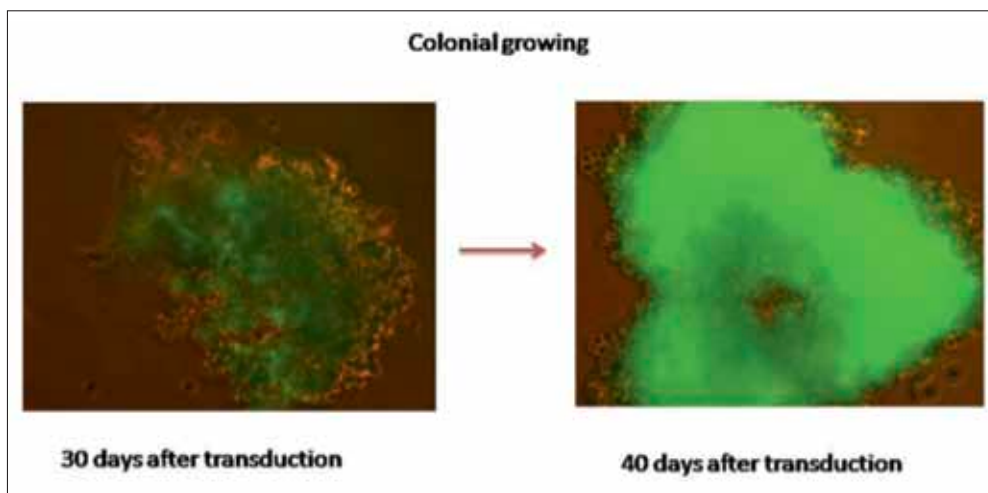
Figure 4. HepG2 hCAR-GFP Stable Cell line transduced with Lentivirus HNF4a and Lentivirus cEBPb cultured in medium supplied with 5 mg/ml blasticidine. Clonal survival was observed, but cells were not able to expand and died some weeks later.

ferentiation. The results showed that it is possible to obtain functional, yet not fully differentiated hepatocytes, as shown by the formation of polygonal colonies, the reversible induction of CYP1A1 gene expression, the induction of onset of polarisation (ZO-1 tight junction protein), and the acquisition of hepatocyte-like phenotype.

The fourth approach (partner VUB), using postnatal progenitor cells as source for the in vitro production of functional hepatocytes has led to the establishment of optimised isolation protocols to obtain rat and human mesenchymal progenitor cells (MPC) from the adult bone marrow and human skin progenitors cells (SKP) from adult dermis. The isolation protocol of human adipose tissue stem cells (ADSC) from liposuction material and adult abdominal fat needed further optimisation to increase the isolation efficiency. Human MPC were able to differentiate into cells expressing a hepatic phenotype and functionality, upon sequential exposure to hepatogenic factors mimicking the secretion pattern during in vivo hepatogenesis and co-exposure to 1μM TSA

Intestine-Liver Co-Cultures

Experiments of co-culture of human intestinal Caco-2 and human hepatic HepaRG cells were performed in collaboration between partners INRAN and INSERM to obtain an in vitro model that could combine absorption and metabolism of nutritionally or pharmacologically relevant compounds in the two coupled systems, to better reproduce the in vivo route of ingested compounds. Caco-2/TC7 were grown and differentiated on filters and HepaRG cell lines grown on the bottom of the culture wells were added in co-culture for the 24 hours of the experiment.
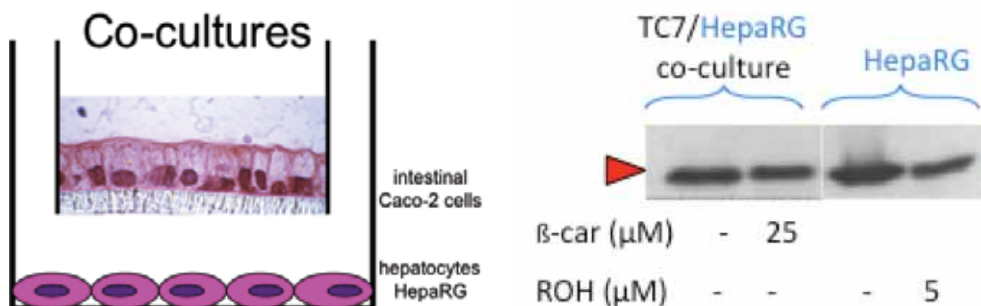
Figure 5. Co-culture set-up.

Functionality of the co-culture model was assayed by measuring beta-carotene and retinol transport and metabolism using as end-point the retinol-dependent secretion of retinol binding protein (RBP) from hepatocytes.

Beta carotene, after partial transformation in retinol by intestinal Caco-2/TC7 cells, was secreted and transported into the basolateral medium in contact with the hepatic HepaRG cells, thus stimulating secretion of retinol binding protein (RBP) bound with retinol (RBP-ROH). Conversely, beta-carotene had no effect when directly applied to hepatic cell cultures, indicating that intestinal metabolism is crucial for the response of the hepatics cells. In conclusion, this preliminary data suggest that this *in vitro* model can be considered a good tool to analyse the absorption and metabolism of retinoids and could be extended to investigations on other dietary compounds and molecules of pharmacological interest.

Intestinal Cell Model

The most frequently utilised intestinal cell culture model is the human colon carcinoma cell line Caco-2. This cell line displays, in long-term culture, several characteristics of differentiated small intestinal enterocytes, such as morphological polarity, development of apical brush border, expression of digestive enzymes, absorptive and metabolic competence. However, the Caco-2 cell lines used in various laboratories exhibit great variability in the expression of differentiated functions, probably due to differences in cell origin, maintenance and assay protocols. Thus a few Caco-2 models differing in origin and maintenance were compared at the level
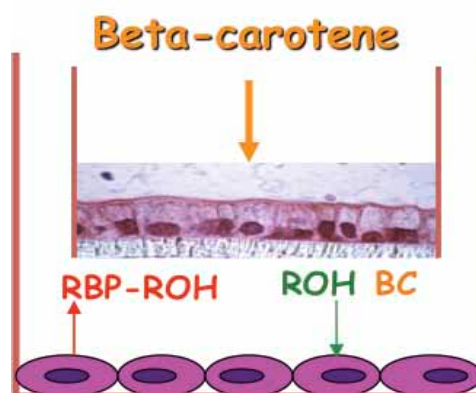

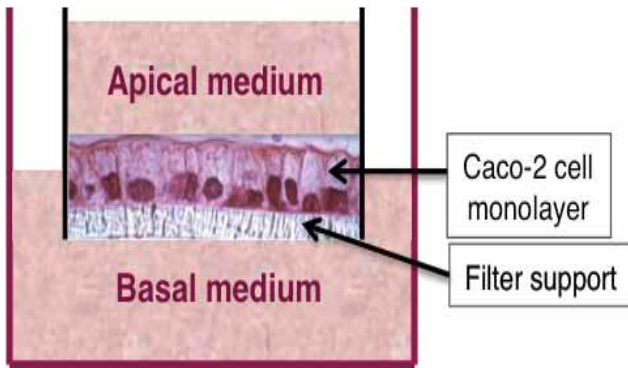Figure 6. Beta-carotene biotransformation in co-cultures.

Figure 7. A. Schematic view of differentiated Caco-2 cells on filter. B. Confocal microscopy of F-actin fluorescent localisation of F-actin: orthogonal and cross-section of the cell monolayer.

of gene expression protein synthesis, and expression of functional activities. Principal component analysis demonstrated that culture conditions are the most important factor affecting differentiation (P1 CNR and P2 EOC/IOSI). Thus, some partners in the project concentrated on the optimisation of culture conditions to improve the reproducibility of this cell culture model.

Caco-2 cell lines of different origin and maintenance protocol were compared by P2 (EOC/IOSI) at the level of gene expression with other tumour intestinal cell lines, namely T84, HT29, SW480 and LS174T, and with normal human intestinal tissue to characterise the degree of expression of genetic markers typical for different regions or cytotypes along the intestinal tract. Caco-2 cells clustered closer to normal enterocytes than any other cell line investigated. It was also shown that Caco-2 cells upon differentiation gained small intestinal traits rather then lose tumoural features. Some data indicated that CDX2 may be a key player in this differentiation process. This is important with respect to the possibility of regulating this process

by external or internal factors.

P1 (CNR) developed a new culture protocol for Caco-2 cells, obtaining a higher degree of differentiation compared to the standard culture conditions. Using
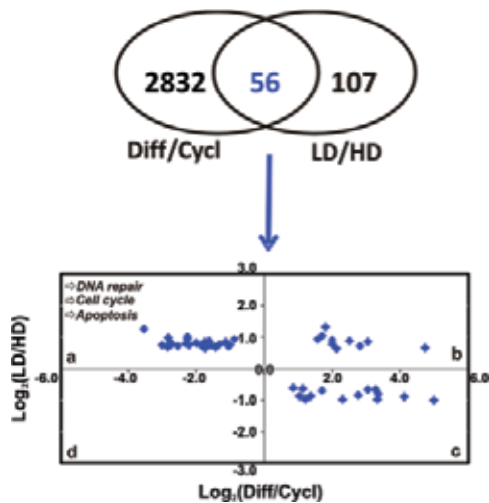

Figure 8. From normalised microarray data a total of 2888 genes ('Diff/Cycl' experiment) and 163 genes ('LD/HD' experiment) were selected, showing an average relative ratio greater than 1.5 or smaller than 1/1.5 in the linear scale. The relative Log2 ratios of genes belonging to both sets, {Diff/Cycl ∩ LD/HD} in blue in the Venn diagramme, are shown in the scatter plot.
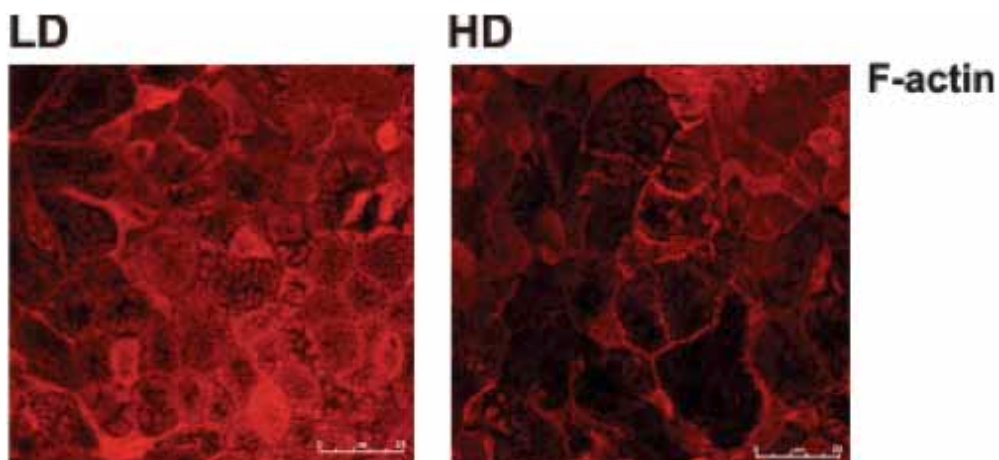
Figure 9. Phalloidin-TRITC staining of F-actin of 21-days differentiated LD and HD cells. Each image is the orthogonal maximum projection of a 100-frames z-stack.

this new culture method, Caco-2 cells expressed high levels of many of intestinal epithelium differentiation markers, as judged by microarray gene expression profile analysis and confirmed by quantitative real-time PCR. Comparing the LD (sub-cultured at low density) and HD (sub-cultured at high density) after differentiation, a list of 163 genes was obtained whose level of expression was different in the two conditions Out of these 56 genes also showed a decrease or increase at least 1.5 folds in differentiated LD cells versus differentiated HD cells. We analysed these genes by plotting Log2 of the two sets. The scatter plot shows both the extent and direction of gene expression variations. Interestingly, the other 50% of the genes in common between the two sets form a cluster only in the (a) quadrant, and include those that were more expressed in LD cells and down-regulated during differentiation. Among these genes most are related to apoptosis, cell cycle and DNA repair.

In addition, the LD protocol confers to the Caco-2 cells a homogeneous and good morphological development of brush border and tight junctions (Figure 9)

Partner INRAN investigated other aspects for the optimisation of the differentiation of Caco-2 cells, including the effects of culture substrate and of medium composition. Caco-2 cells require two to three weeks of confluence to achieve optimal morphological polarisation and expression of differentiated traits. The permeability of the culture substrate (impermeable plastic or glass versus permeable filter) was shown to strongly affect the differentiation process. The expression of several differentiation-related genes was 2 to 3 folds higher in Caco-2 cells differentiated on filter than on plastic. Filter-differentiated cells were morphologically more homogeneous, expressed higher enzymatic activities and showed better localisation of the tight junction protein claudin 4 to

Gene expression of filter-differentiated Caco-2 at day 15 compared to cells on plastic

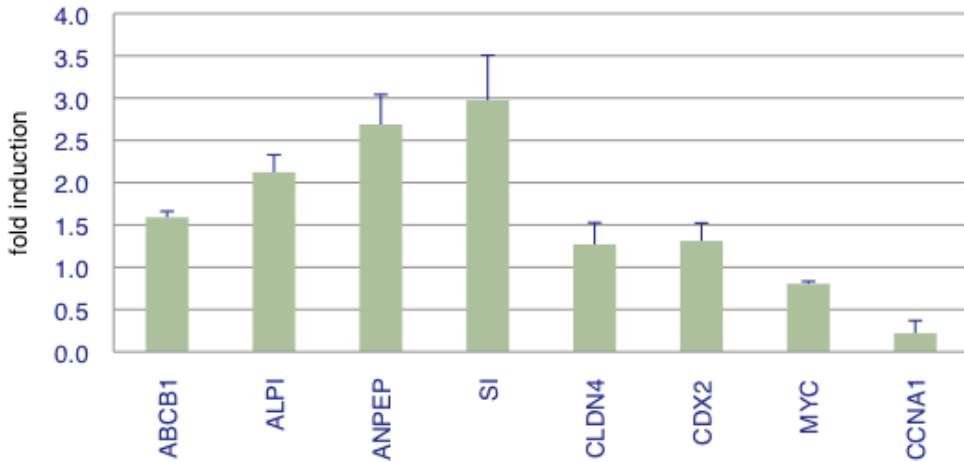Figure 10. A. Expression by RT-PCR of genes coding for apical Pgp transporter (ABCB1, enzymes aminopetidase N (ANPEP), alkaline phosphatase (ALPI) and sucrase (SI), tight junction protein claudin 4 (CLDN4), transcription factor CDX2 and oncogene MYC. All were normalised against the house-keeping gene PP1A. B. Immunolocalisation of claudin 4 in plastic vs glass differentiated Caco-2 cells at day 21 from seeding.
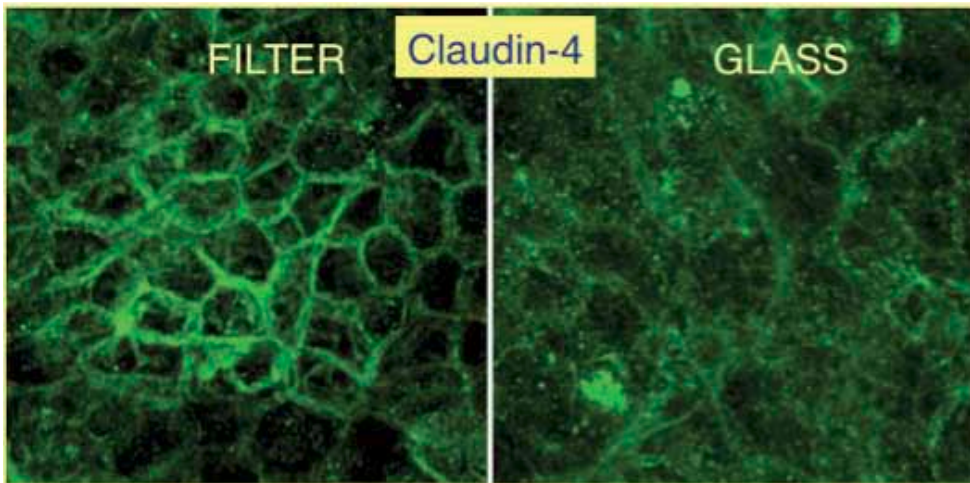
Table 1. Values are expressed as percent of control cells differentiated in medium with 10% FBS in apical and basal compartments (control). Mean of 3-4 experiments performed in triplicate.

Parameters Measured at Day 21 from Seeding	10% FBS in Basal Medium	
% of control	MITO+ serum extender in Basal medium	
% of control		
Permeability (mannitol passage)	99.24	177.88
PGP activity (BL to AP digoxin)	144.35	158.23
PEPT1 activity (cephalexin AP to BL transport)	105.75	202.39
Alkaline phosphatase activity	90.19	62.43
Sucrase activity	104.41	41.24

the cell border, compared to cells differentiated on plastic or glass.

INRAN also tried a chemically defined culture medium without fetal bovine serum (FBS) supplementation to reduce the variability introduced by the variable and unknown composition of serum batches employed for cell culture media. The replacement of FBS from the culture medium with a chemically defined supplement (MITO+ serum extender) allowed a good degree of differentiation of the cell line under better controllable and reproducible conditions. In particular, optimal development of barrier properties (mannitol passage) and expression of active transport activities (PGP and PEPT1) was obtained in chemically defined medium. Lower enzyme activities (alkaline phosphatse and sucrase) were detected in serum-free medium. In addition, it was shown that even when FBS was used in the culture medium, its presence in the apical medium was

not necessary to allow optimal functional differentiation, as shown by all parameters investigated (Table 1). Unfortunately, the exact composition of the MITO+ serum extender is not released by the manufacturer, thus reducing its potential as an optimal 'chemically defined' medium, as its reproducibility rests upon its production quality. It can therefore be concluded that FBS can be replaced by MITO+ serum extender in the basal medium during differentiation of Caco-2 and in any case, even if FBS is used, it should be omitted from the apical medium to reduce its costly and unethical use.

Transport Activities

Since the aim of the project was to obtain a well-characterised cell line representative of human small intestinal enterocytes, expressing absorptive and metabolic functions, another set of studies dealt with the characterisation of the transport activi-

EFFLUX

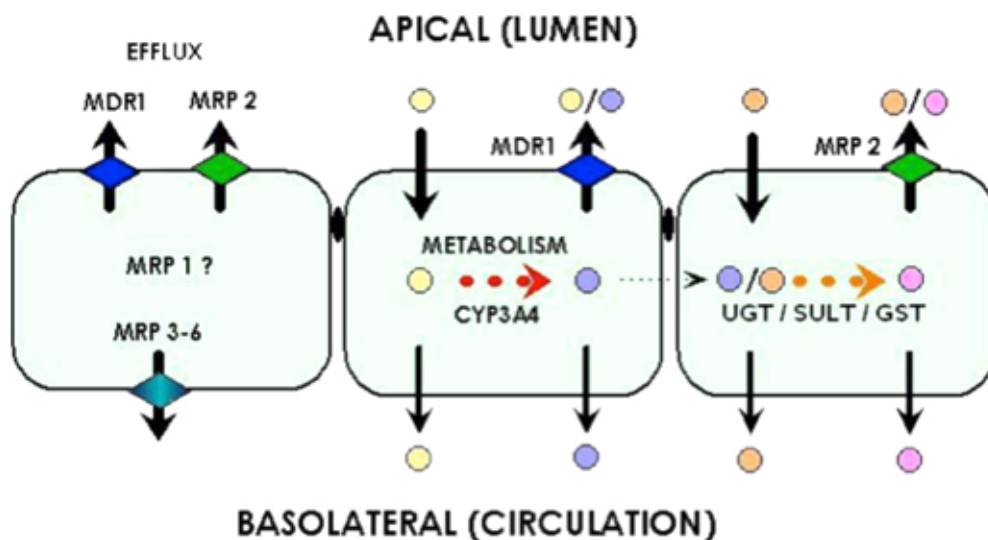**APICAL (LUMEN)**

BASOLATERAL (CIRCULATION)

Figure 11. Potential patterns of interplay between the most important drug efflux and metabolism processes observed during the Caco-2 permeability studies. Localisation of the efflux proteins are presented as diamonds and drug molecules as circles; parent drugs in yellow and orange, phase I metabolites in blue and phase II metabolites in magenta. BCRP is also localised in the apical membrane of the Caco-2 cells and may contribute to the transport of phase II metabolites. (Sanna Siissalo, 2008)

ties expressed by the Caco-2 parental cell lines, the clonal Caco-2/TC7 line and the patented model CacoReady (objectives 2 and 3). Permeability and transport experiments were performed using the same set of FDA recommended model drugs and the same experimental conditions commonly agreed by all partners involved. Permeability was assessed with mannitol (Low permeation), atenolol (Intermediate permeation), metoprolol (High permeation). The active uptake was measured using the PEPT1 substrate cephalexin, and active apical efflux by using digoxin (+/- verapamil as specific PGP inhibitor). Kinetic analysis of active apical efflux of digoxin by PGP was also performed in some selected Caco-2 cell lines.

In addition, P6 (HY.FL) in collaboration with P10 (Novamass) tested more than 20 compounds representative of different rates and mechanisms of transport (low and high passive permeation) and PGP efflux substrates. Automation with Tecan Genesis provided a proven methodology of cell permeability assays in a high-throughput mode.

The tiered approach by WP5 was taken into account to provide high quality data for the kinetic modelling. These investigations are leading to two peer reviewed publications to give the scientific community first hand aid and recommendations for cell permeability testing protocols.

The transport activity experiments per-

formed with mannitol, atenolol, propa-nolol, cephalexin and digoxin produced several indications concerning the perfor-mance of the various models.

Comparison of transport of selected drugs in Caco-2/INSERM and Caco-2/TC7 gave comparable results (Figures 12 and 13) also indicating good expression of the two active transporters (PGP for apical ef-flux of digoxin and PEPT1 for cephalexin transport).

Data set of transport properties of CacoReady 96-HTS or 24-HTS format has been produced showing a better performance of the latter system. Trans-port properties of CacoReady 96-HTS and CacoGoblet 96-HTS were also ana-lysed. Although PGP-specific apical ef-flux was diminished in CacoGoblet cells, CacoReady and CacoGoblet cell models presented similar levels of digoxin trans-port. CacoReadyTM presented higher PGP activity that makes it more suitable for identification of PGP-dependent com-pounds (partner Advancell).

Limited to the role of human MDR1, an alternative cell line of MDCK cells trans-fected with PGP transporter, has been evaluated as a tool to discriminate PGP substrates from non-PGP substrates. An assay has been automated and used to as-sess permeability, role of PGP and poten-tial of inhibition of a selective substrate (digoxin). A PGP (ABCB1) vesicular trans-port system and PEPT1 and OCT1 uptake transport assays have been developed. Optimised protocols to screen potential substrates of selected efflux proteins for higher-throughput methodology have been produced (P15 SOLVO).

In the HepaRG cells transporter studies have shown that these cells are equipped with OCT, NTCP and the efflux transport-ers, that NTCP function is much higher than expected from the level of mRNA, that OATP 1B1 is low and other OATPs are present (P5 INSERM). These cells can therefore be recommended for efflux stud-
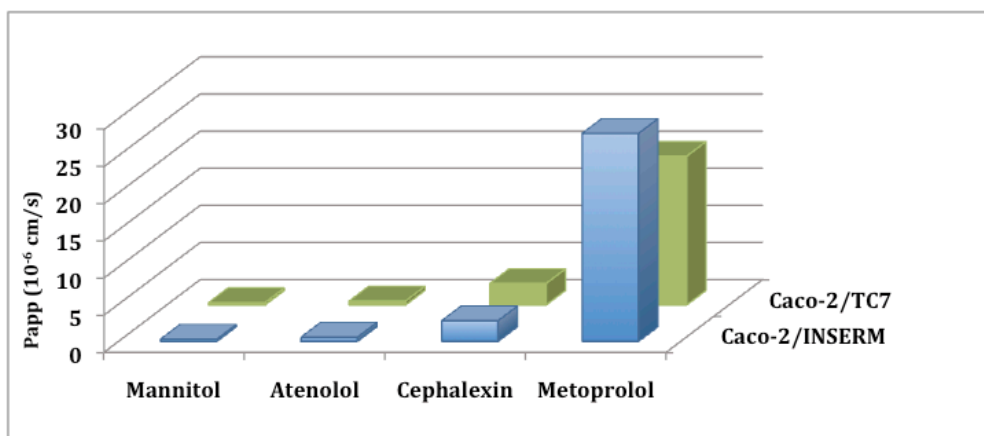

Figure 12. Apical to basolateral transport of model drugs selected on the basis of their permeation properties in Caco-2/INSERM and Caco-2/TC7 cells. (Partners INRAN and Merck).
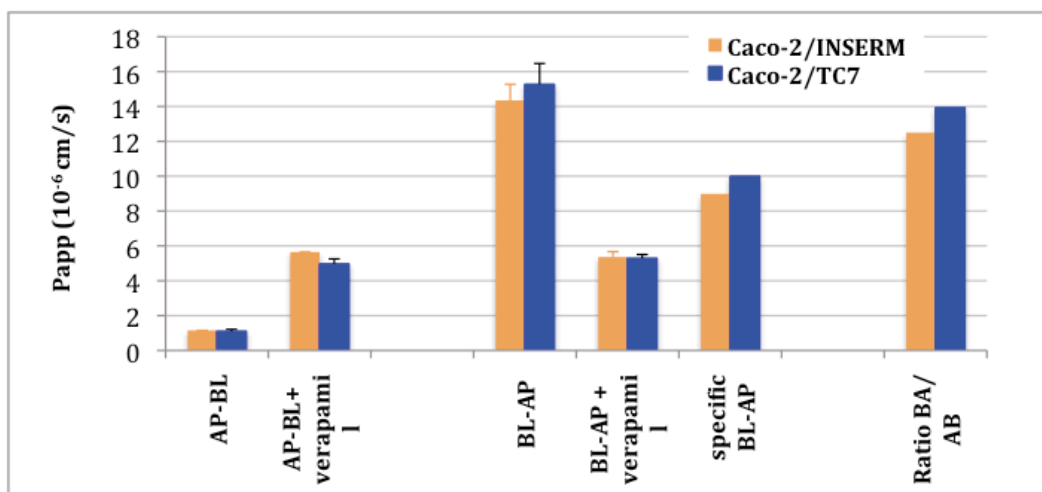
Figure 13. Digoxin transport in both directions was performed in to determine the asymmetry index (Ratio BA/AB) and the specificity of efflux by PGP by using the selective inhibitor verapamil.

ies in a much simpler context than the sandwich system.

Concerning metabolic activities, PCR primers have been supplied by partners BPI and Novomass and HYFL for 23 human CYPs, and a set of selected compounds for probing UGT activity in intestinal samples has been set up. The genomic profiling of some selected transporters along the human intestine and data mining of publicly available data sets has confirmed the presence of the relevant transportes in the Caco-2 cell line. In Caco-2 cells it has been shown that differentiation has a stimulatory effect on UGT glucuronidation activity. In normal intestine the glucuronidation of estradiol 3-G is particularly effective, while this is not the case in Caco-2 cells, which express high levels of UGT1A6, also in the undifferentiated state. It has also been shown that pH differences (pH 6.0 vs. pH 7.4) affect glucuronidation activities (P6

HYFL).

The compounds selected for the characterisation of Caco-2 and MDCK-MDR1 cell monolayers were tested for drug transporter interactions on membrane and cell-based assays to validate their selectivity or their role as passive controls (P13 SiBi).

Toxicity Detection

Concerning the toxicity approach (P3 HU-LAFE), a multiparametric analysis of xenobiotic toxicity at the level of individual cells using flow cytometry and cellular imaging-based approaches, such as high-content analysis (HCA) technology, could play a key role in the detection of toxicity and classification of compounds based on observed patterns of cellular injury. The aim of the study was to develop and validate a practical and reproducible *in vitro* multiparametric cell-based protocol to assess human hepatotoxicity potential of drugs
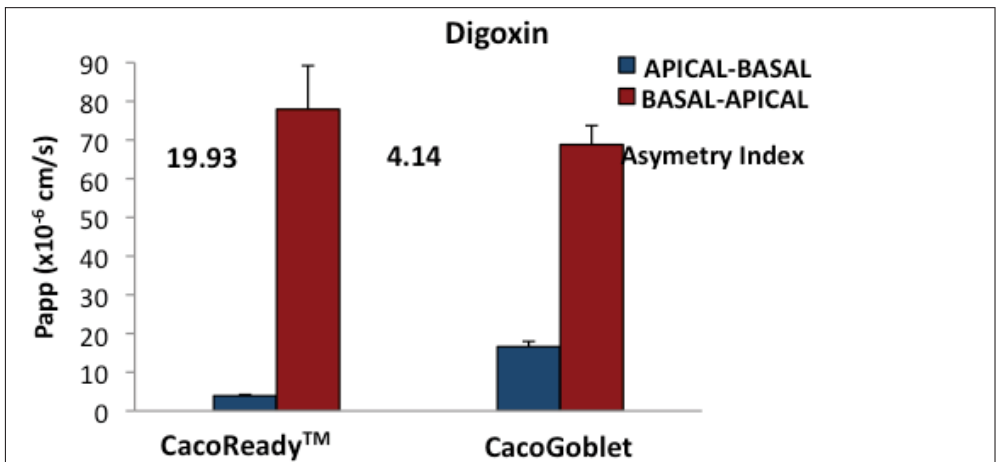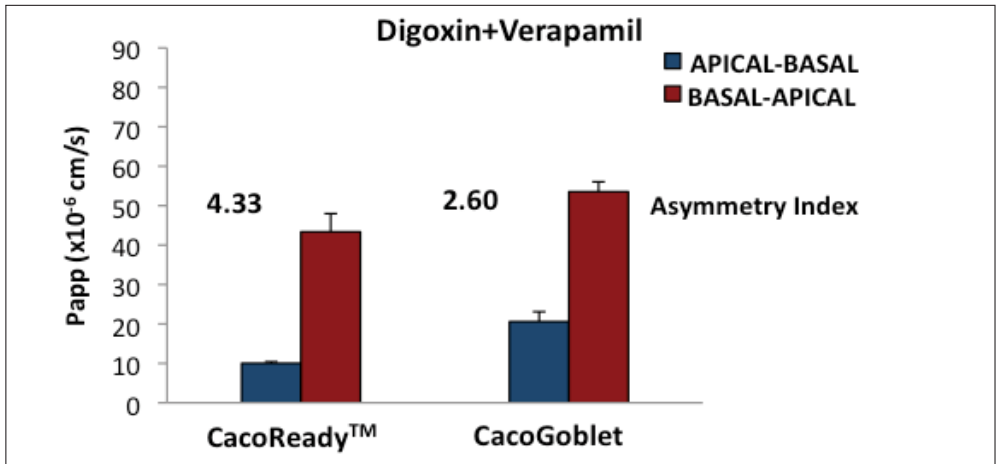
Figure 14. Comparison of Digoxin and Digoxin+Verapamil efflux in CacoReadyTM and CacoGoblet cellular models.

and classify them by their mechanism-of-action. The assay was applied to HepG2 human hepatoblastoma cell line cultured in 96-well plates and exposed to 40 different compounds for 3 and 24 hours at a range of increasing concentrations (1-1000 µM). Cells were loaded with five fluorescent dyes showing optical compatibility for mitochondrial membrane potential (TMRM), lipid peroxidation (BODIPY 665/676), intracellular calcium concentration (Fluo-4 AM), DNA content (Hoechst 33342) to determine nuclear area and cell number and viability (propidium iodide), and then analysed in the High-Content Screening Station Scan^R (Olympus). In another assay three probes: a-GST release, 8-ISO (phospholipids peroxidation) release and 8-oxodoG (DNA peroxidation) release were assessed to investigate the oxidative stress. The assay used citoculline as negative control.

Figure 15. Functional activity of efflux transporters in differentiated HepaRG cells.

Figure 16. Multiparametric assessment of toxic compounds.

By using the new technology of HCA a re-producible high-content multiparametric cytotoxicity assay was set up, based on the measurement of 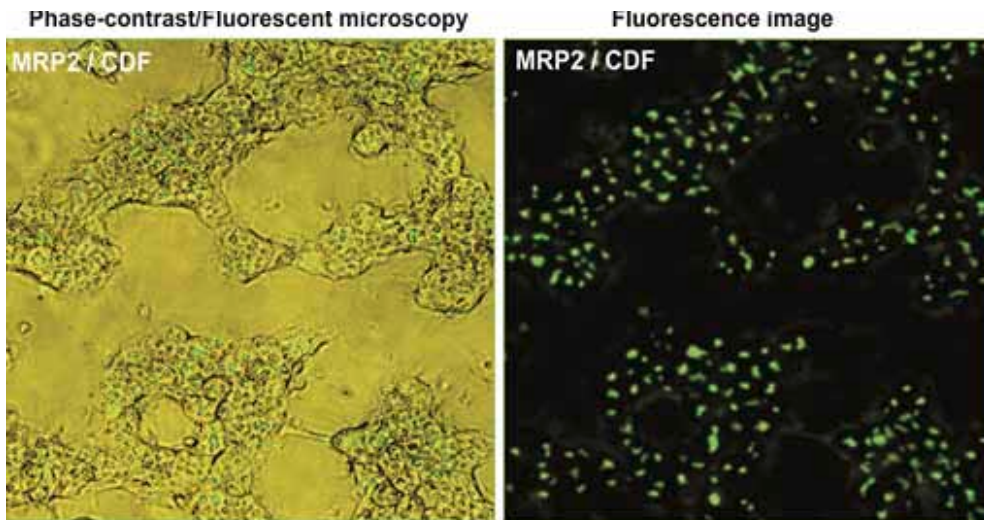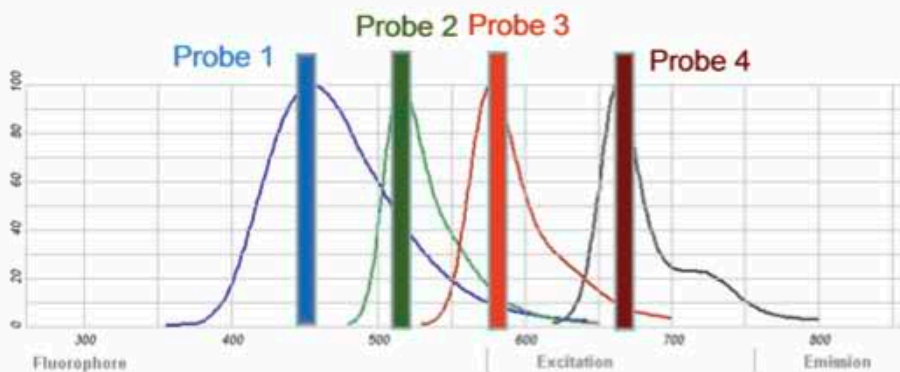multiple parameters that are morphological and biochemical indicators of prelethal cytotoxic effects at the level of single cells and allows a high-throughput screening. This strategy seems to identify early and late events in the hepatotoxic process, and it allows classification of compounds according to their mechanism-of-action and their toxic potential (non-toxic, low, moderate, and highly toxic). A list of model hepatotoxins representative of the different molecular mechanisms of hepatotoxicity has been elaborated. A subset of them, supposed to increase oxidative stress (17 compounds) was then evaluated in this system. As shown in Table below, at least one of the parameters was affected by oxidative stress inducers. Exceptions were

represented by compounds that need bio-activation to initiate the toxic effect, e.g., acetaminophene, representative of different mechanisms of toxicity.

A suitable mathematical and statistical analysis of the data is needed in order to classify unknown compounds in the future (Principal Component Analysis). If predictive cellular systems can be developed and applied to identify a significant number of hepatotoxic drugs with a high degree of specificity, it would undoubtedly improve the safety profile of new therapies and impact the well being of both humans and animals. Partners INSERM, VUB, and SiBi participated in this effort.

Partner CNR tested an acute treatment with two toxic concentrations of copper chloride on cells maintained with LD and HD protocols. Copper treatment resulted in different levels of actin depolymerisa-

Table 2.

Compound	GSH LOEL (uM)	8-oxodG LOEL (uM)	8-Isopr LOEL (uM)	Alpha GST LOEL (uM)	No. Ox stress markers modulated	MoT
Tamoxiphen	12.5	-	-	100*	1	OS
Acetaminophen	-	-	-	100	0	OS
Clofibrate	-	-	-	100	0	M
Citicoline	-	-	-	100*	0	-
Mevinoline	25	-	-	100	1	OS
Isoniazid	-	12.5	12.5 (v)	100	2	OS
Acetylsalicylic acid	-	-	12.5 (v)	-	1	OS, M
Rotenone	50	-	6.25	-	2	OS
Pentachlorophenol	100	100	12.5	-	3	OS
Azathioprine	-	100	12.5	-	2	OS
Valproic acid	-	-	12.5	-	1	OS
Erythromycin	-	-	1.56	-	1	OS
Menadione	6.25	100	6.25	-	3	OS
Colchicine	100	12.5	1.56	-	3	OS,M
Chloramphenicol	-	12.5*	6.25	-	1	OS
Nitrofouratoin	-	12.5	12.5	-	2	OS
Thioacetamide	25	100*	25	-	3	OS

	Viability		Nuclear changes				Morphol. changes
MORPHOLOGICAL	Cell number	Alive cells	Nuclear area	Nuclear elongation factor	Nuclear compact.	Nuclear circularity	Cell area, compact.
FUNCTIONAL		Cell compactness	Nuclear elongation factor	Nuclei/ cell intensity	Fluo4 intensity	TMRM intensity	BODIPY intensity
	Apoptotic cell death				Intracel. Ca^{2+}	Mitoch. impairment	Lipid perox.

tion and gene expression induction relative to the culture protocol, the LD cells showing a more homogeneous and stronger toxic response. These results indicate the importance of the maintenance protocol on the physiological response of the cells. The HD and LD differentiated cells displayed a number of morphological and physiological differences that must be taken in account when these cells are used as an intestinal model.

In Silico Modelling

The *in silico* modelling activities (partners UM, Symcyp, and SiBi) relating to objective 4, resulted in a tiered approach for the assessment of the pharmacokinetic models. Four levels were proposed, which were relevant to both the selection of probe compounds and the conduct of the appropriate experiment that would allow evaluation. Different systems were deemed appropriate for different purposes (hence the need for different levels of evaluation). The objective of the first level was to triage novel systems based on existing information generated using whatever methods are currently available in their own laboratories. At the second level, metabolic and transporter competence and cellular in-

tegrity were assessed qualitatively. It was expected that at the third level, full kinetic studies would be performed using specific probes under standard conditions of linearity. The fourth level represented the most detailed level of evaluation involving several model compounds that had *in vivo* correlates available to be used in the assessment.

A list of compounds has been worked out both for permeability and for metabolism parameters. State-of-the-art models such as human cryopreserved hepatocyte were compared with a new model developed in the project: differentiated HepaRG cryopreserved cells.

Three lists of model compounds (each 30-50 drugs) for intestinal permeability, hepatic and intestinal metabolism were collated from the literature. The choice covered a wide range of: permeability/metabolic clearance values, physico-chemical properties and transport mechanism/enzyme involvement. In addition a much shorter list of 'first choice' drugs (calibrators) for metabolic study was also provided. Models for prediction of intestinal permeability, hepatic clearance and Fg (fraction escaping first pass metabolism
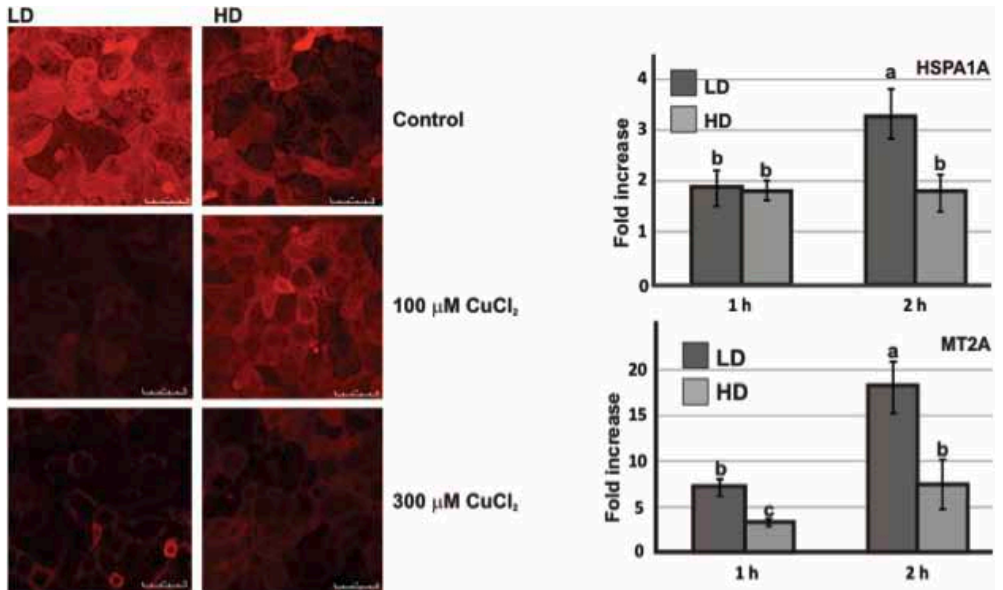
Figure 17. (Left panel) Phalloidin-TRITC staining of F-actin of 21-days differentiated LD and HD cells treated for 2 hours with increasing concentrations of copper chloride. Each image is the orthogonal maximum projection of a 100-frames z-stack. Scale bar: 25 micron. (Right panel) Relative expression by quantitative RT-PCR of two genes involved in the response to copper toxicity, MT2A and HSPA1A, in 21-days differentiated LD and HD cells treated with 100 μM CuCl2 for 1 or 2 hours. The values are expressed as fold induction with respect to untreated LD or HD differentiated cells. Different letters above error bars indicate significant (P < 0.01) differences between groups calculated by one-way ANOVA followed by Tukey's post-hoc test.

Figure 18.
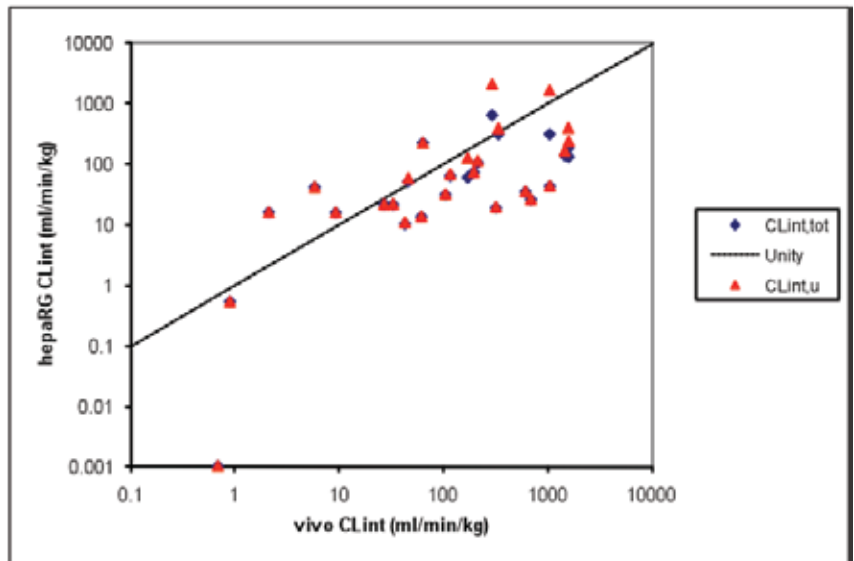
in the gut) that were available in the literature were also provided.

Level 4 of the tiered approach was reached only in a limited set of models. Permeability in *in vitro* systems was not easily connected with the Fraction absorbed measured in clinical studies. On the other hand, HepaRG resulted in prediction very close to that of pooled human primary hepatocytes. Moreover, the quality of prediction was as good as that obtained with hepatocytes. This suggest that HepaRG can represent a valid surrogate of pooled human primary hepatocytes, with the advantage to be more consistent and reproducible.

Conclusions

An optimised *in vitro* model of hepatocytes is now available, well characterised for different functions and for the response to specific toxicants: the HepaRG cell line. The project has also supported new lines of research for obtaining functional hepatocytes *in vitro*, in order to promote the availability of more than one *in vitro* model, thus amplifying the opportunities. The transfection approach with multiple factors inducing the expression of major metabolic activities has been successfully accomplished; however the procedure only succeeded in Hela cells but not in hepatic cell lines. The TSA treatment to induce hepatic differentiation has proved effective in human mesenchymal progenitor cells, for which isolation protocols have also been set up.

The intestinal model that has shown to be the most similar to normal small intestinal enterocytes *in vivo* was the Caco-2 cell line. These cells have been characterised at genomic and functional levels. Differentiated Caco-2 cells seem the model of choice for oral drug absorption, especially for active uptake, less for secretion. An optimised method of maintenance of the cells has been set up, which allows a strict control of the differentiation process. This approach has shown that source of the cells or the number of passages (e.g., sub-cultures) are not so critical if the culture procedure is strictly followed. Other aspects have been investigated in relation to the optimal expression of the phenotypic and genotypic markers: cell differentiation on permeable substrate (i.e., filters) is the only one that guarantees the correct polarisation and differentiation of the cells. Moreover, since the serum is still a factor of variability and interference especially in toxicity testing, synthetic media have successfully been tested and it has also been also shown that serum addition is not needed in the apical medium.

Concerning transport and metabolic activities, while they have been well characterised in the cellular models used in the project, it has not been possible to assess them with respect to a wider choice of compounds, since the extensive characterisation of the models has requested more time and work than expected. That has been also the reason for the request for six-month extension of the project.

This situation has also had an impact on the *in silico* modelling work, as only a

limited amount of data could be provided to partners UM and Simcyp. However, an improved strategy and recommendations on the requirements for accurate determination of permeability data for *in vitro - in vivo* correlation and *in vitro - in vivo* extrapolation have been produced. The third stage (single compounds to be tested in order to produce comparable quantitative kinetic data) has only been partially performed due to the difficulties in setting up experiments. Thus, the final elaboration has not provided the predictive value of the selected *in vitro* models with respect to the *in vivo* situation.

Toxicity studies on hepatocytes have been extensive, based on new fluorimetric approach and a number of selected compounds, the list of which has been published to the benefit of a wider scientific audience. On intestinal cells mainly comparison of toxicity, in different types of culture conditions, have been performed in order to evaluate the new culturing procedure.

The LIINTOP project has achieved the goal of proposing two well characterised reliable in vitro models for the evaluation of xenobiotics/drugs absorption, metabolism and toxicity in liver and intestine: the HepaRG cells in the case of liver, and the Caco-2 cells for the small intestine. Those models are ready for validation and thus available for the assessment of their predictivity versus the *in vivo* situation.

Extensive characterisation has indicated that many of the functions are consistently similar to those present *in vivo*, thus a good agreement may be expected from follow-up studies. Other functions have been described, which do not completely overlap to the corresponding *in vivo* ones. This elucidation is relevant too, addressing the limitations of those models.

This latter aspect justifies the research performed in the LIINTOP project, on setting up other *in vitro* models, following different strategies:

- In the case of hepatocytes: transfection with regulating factors of hepatic functions; the use of HDAC inhibitors to induce differentiation in primary culture of hepatocytes or of stem cell. All these approaches have produced an advancement of the knowledge of the specific *in vitro* models and the optimisation of appropriate technique.

- In the case of intestinal cells: CYP transfection of Caco-2 cells has been tried, but the efforts were unsuccessful.

The case of intestinal cells is very relevant, because the intestine is a first pass-barrier for several drugs or ingested contaminants. Already at this level the compounds can be metabolised and/or be toxic. Unfortunately the many attempts performed over the last 20 years, for isolating and maintaining in culture normal enterocytes, have all been unsuccessful: it would be appropriate in the future to attempt production of differentiated intestinal cells from stem cells (possibly human) in order to produce other *in vitro* models to be used for studies of absorption and metabolism.

Dissemination

The dissemination of the outcomes of the project has been very effective and successful: up to now 103 oral presentations (some of them on invitation) have been given by the partners. 42 posters have been presented at international meetings and 75 papers have been published in peer reviewed scientific journals. It is worth noting that, in addition to publications, also the other diffusion activities had a world-wide dimension.

Concerning the exploitable knowledge at the level of industrial research, partners INSERM (UR), BPI and UM have improved and better characterised the cellular model HepaRG already protected by a patent.

Moreover, partner SiBi has produced a methodology to measure a panel of markers for oxidative stress, while partners Advancell and Solvo have set up a PreadyPort platform for measuring MDR1 and BCRP (cellular transporters).

Co-ordinating partner CNR has produced a protocol that allows a synchronised differentiation of the Caco-2 cells, which can be used widely to obtain better-differentiated cells, comparable across laboratories and thus more standardised.

The dissemination activities will continue, since other papers are either submitted for publication or in preparation. Other invited presentations are also scheduled in the near future for some of the partners.

In addition to that, the Co-ordinator has already contacted the Editor of *Toxicology In Vitro* to ask about the possibility of having an issue of this journal devoted to the LIINTOP project. The request had a positive answer and it is planned to present a proposal, agreed upon by the partners, in autumn 2010.

Finally, a series of document are available as protocols for specific methodologies or as theoretical elaboration on appropriate strategies to improve the production and elaboration of data for functional evaluation (both at the qualitative and quantitative level) of the cellular models.

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NanoTEST

Development of Methodology for Alternative Testing Strategies for the Assessment of the Toxicological Profile of Nanoparticles Used in Medical Diagnostics

Contract number: Grant Agreement 201335
Project type: Specific Targeted Research Project (FP7)
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Starting date: 1 April 2008
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Website: nanotest-fp7.eu

Background

Nanoparticles (NPs) have unique, potentially beneficial properties, but their possible impact on human health is not known. The area of nanomedicine brings humans into direct contact with NPs and it is essential for both public confidence and nanotechnology companies that appropriate risk assessments are undertaken in relation to health and safety. There is a pressing need to understand how engineered NPs can interact with the human body following exposure. Additionally, it is important to develop alternative *in vitro* and *in silico* testing strategies specifically for nanomaterials. The FP7 project NanoTEST addresses these requirements in relation to the toxicological profile of NPs used in medical diagnostics.

A better understanding of how properties of NPs define their interactions with cells, tissues and organs in exposed humans is a considerable scientific challenge, but one that must be addressed if there is to be safe and responsible use of biomedical NPs. NanoTEST will evaluate toxic effects and interactions of NPs with biological systems used in nanomedicine. There are a number of different NP characteristics, which will influence transport and toxicity including size, surface area, coating and charge. With the use of a suitable panel of NPs of the highest purity, we will determine how these characteristics relate to possible adverse health effects and develop *in vitro* test methods and testing strategies for NPs toxicity testing.

Objectives

The overall aim of this project is to develop alternative testing strategies and high-throughput toxicity testing protocols using *in vitro* and *in silico* methods which are essential for the risk assessment of these NPs. To be able to achieve this ambitious goal, the specific aims of NanoTEST are as follows:

a) to carry out a detailed characterisation of selected NPs in order to define their main physico-chemical properties
b) to study specific and nonspecific interactions of NPs with molecules, cells and organs and to develop *in vitro* methods which can identify the toxicological potential of NPs
c) to validate *in vitro* findings in short-term *in vivo* models, to study manifestation of particle effects in animals and humans, and to assess individual susceptibility in the response to NPs
d) to perform both structure-activity modelling and physiologically-based pharmacokinetic (PBPK) modelling of NPs; and
e) to adapt the most advanced and promising assays for high-throughput automated systems and to prepare for validation by the European Centre for the Validation of Alternative Methods (ECVAM).

NanoTEST integrates the investigation of toxicological properties and effects of NPs in several target systems by developing a battery of *in vitro* assays using cell cultures, organotypic cell culture derived from different biological systems: blood, vascular system, liver, kidney, lung, placenta, digestive, renal and central nervous systems. As the activity of NPs is likely to involve oxidative stress we will focus on the cross-cutting areas of inflammation, cellular toxicity, immunotoxicity, genotoxicity and related endpoints. Following development of Standard Operating Procedures (SOPs) and generation of a common database, and in parallel with *in silico* assays (QSAR, PBPK modelling), NanoTEST will evaluate toxic effects and interactions of NPs used in nanomedicine. Results will be validated in an experimental ethically approved *in vivo* model. The most advanced and standardised techniques will be adapted for automation and prepared for validation by JRC (ECVAM). Finally, we will propose recommendations for evaluating the potential risks associated with new medical NPs, which will be communicated to the scientific and industrial community.

Deliverables & Milestones Achieved During 2010

The overall aim of NanoTEST is to develop new testing strategies for NPs used in medical diagnostics. To achieve this aim, it was absolutely crucial to have experimental data from all tested NPs to be able to develop assays and adapt them for testing NPs. These results are important also for selecting the most promising assays to be part of bathery for test strategy. The deliverables for 2010 (Tables 1-2) are mostly focused on characterisation, toxicity testing in representative cells

Table 1. Deliverables achieved in 2010. Several deliverables are constantly updated to complete all nanoparticles. Numbering is according to Grant Agreement.

2010 Deliverables		Status
1.1	Bank of well-characterised NP	√
1.3	Physico-Chemical characterisation of selected NPs	√
2.1.1	Evaluation of the genotoxic & immunotoxic impact of the NP	√
2.2.1	Identify effects of NP on endothelial cells	√
2.3.1	Establish suitable liver cellular models for NP toxicity assay (SOP)	√
2.3.2	Identify hepatocyte endpoints	√
2.3.3	Identify Kupffer cell endpoints	√
2.4.1	Comparison of uptake, oxidative stress cytokine secretions & apoptosis induced by NP on lung cells	√
2.4.2	Determination of the mechanisms of uptake & pro-inflammatory responses & pathways signalling in lung cells	√
2.6.1	NP characteristics in digestive cell models defined	√
2.7.2	Evaluation of effects of NP on microglia	√
2.7.3	Interaction of NP with cerebral endothelial cells	√
2.9.2	Selection of candidate assays for automation	√
4.2	Completion of a simple, compartment-based PBPK model	√

from 8 different organs and tissues and investigating how NPs interact with the biological system. Some achievements are highlighted below.

Considerable progress has been made in 2010 towards development of *in vitro* assays, selecting most promising tests for NPs testing and for suggesting alternative testing strategies for nanomaterial safety. Specifically:

Characterisation

• All reference NPs, the PLGA-PEO polymeric NPs (140 nm, provided by P10 ADVANCELL), uncoated iron oxide NPs (Fe_3O_4 , 8+3 nm core) and oleic iron oxide NPs (both provided by PlasmaChem), titanium dioxide (TiO_2) NPs (provided by JRC, manufactured by Evonik), fluorescent (Rhodamine) silica NPs (size 25 nm and 50 nm) both provided by Microspheres-nanospheres, Endorem (as negative control NPs, Guerbet provided by CHUV) were fully characterised. In addition to basic primary characterisation, advanced primary characterisation for some specific NPs (such as photocatalicity and magnetic properties) has been performed.

Table 2: Milestones achieved in 2010. Numbering is according to Grant Agreement.

2010 Milestones		Status
9	Particle definition & characterisation	√
10	Experiments on target cells	√
11	Definition of relevant cells as reporters for NP effects	√
12	Determination of NP in biological tissues	√

Moreover, a detailed investigation of behaviour (agglomeration, stability, precipitation) of selected NPs in all tested biological media has been carried out. Eventually, specific dispersion protocols for investigated NPs according to project requirements have been developed.

Progress made over the last year: compared to 2 NPs characterisation completed last year, additional 3 NPs have been fully characterised and all missing secondary characterisation in different media had been completed. It also appeared that characterisation methods have to be included into battery of assays. SOPs for most promising characterisation methods had been developed.

In Vitro Screening Tests & Selection: Overview

- In 2010 NanoTEST consortium has generated enormous amount of experimental data. Within WP2 (*in vitro* screening tests) cells from 8 different organs and tissues, for the vascular system, cells from liver, kidney, lung, placenta, digestive and central nervous systems) have been used and exposed to the selected reference NPs using markers of oxidative stress, inflammation and immunotoxicity, cytotoxicity, genotoxicity, uptake and barrier transport. The generated knowledge will be an important basis for the next steps toward a NPs specific test strategy.

- To be able to compare our data, in all experiments, all partners used NPs from the same batch, used the same dispersion protocol and followed the same experimental design 5 + 1 concentrations to obtain dose response data. In all experiments quality controls and standards were used, 3 replicates of experiments obtained at different dates were performed, and 4 time points for treatment (0, 4, 24, 48, and 72 hrs optional for some cell types) were used. Data are completed for most of assays with all NPs. Partners started to use NapiraHUB database. As it is important to compare data across cell types, NPs and toxicity endpoints, it is important to use the same template for uploading raw data to the developed Excel spreadsheet. Data from oxidative stress assays are already uploaded into the database and uploading of finished experiments is in the progress.

Progress made over the last year: We selected most promising toxicity tests and methods to be used for future NPs testing. We developed SOPs using ECVAM-JRC format to be ready for further validation of assays. There are already 27 different SOPs addressing all relevant toxicity endpoints completed (no SOP was completed in last report). Selection criteria for the best *in vitro* methods for NPs testing are among others: relevant endpoints, assay reliability, sensitivity and specificity, no or minimal interference of nanomaterial with assay or possibility to control this phenomenon, robustness and automation, *in vitro - in vivo -* and human data comparison and concordance, etc. All SOPs are uploaded on NanoTEST website.

In Vitro Screening Tests: Uptake & Barrier Transport

- Uptake and Barrier transport have been evaluated for a sub-set of NPs using a selection of appropriate in vitro barrier models for the blood-brain barrier (BBB) (HCEC cells), kidney (MDCK, LLCPK), gastrointestinal barrier (CacoReady and CacoGoblet) and placenta (BeWo). The transport studies of NPs were limited to iron oxide and fluorescent NPs for technical reasons. Iron oxide (oleic acid coated, uncoated NP) and fluorescent silica (25, 50 nm diameter) have been studied as these NPs are easily detectable.
- In the BBB model, uncoated iron oxide

NPs were taken up into the HCEC cells whereas coated iron oxide NP showed very low cell uptake. However there was no evidence of transport of coated or uncoated iron oxide NP across the HCEC monolayer. The uptake of both sizes of fluorescent silica NPs by the HCEC cells has been confirmed by fluorescence microscopy and TEM.
- Evaluation of uptake of NPs by LLCPK and MDCK cells demonstrated an uptake of uncoated iron oxide NPs but no uptake of oleic acid-coated NPs.
- Coated iron oxide and both sizes of fluorescent silica NPs transfer to a limited extent across a BeWo placental cell barrier. Uncoated iron oxide NPs agglomerate very quickly and do not cross the cell barrier. Coated iron oxide NPs do not adhere strongly to the cells whereas uncoated iron oxide and both silica types accumulate in the BeWo cells in a dose-dependent manner.
- The uptake by cells of solid core NPs was determined by TEM for cells of different origins. Fluorescent silica and iron oxide NP uptake by cells was also determined using fluorescence microscopy and Prussian Ble reaction, respectively, in the same cells. Uptake could be demonstrated for all NPs evaluated, and the cellular consequences of this uptake are presently being analysed.

Progress made over the last year: Uptake of NPs by cells and transport was not originally included into project but during the course of project it appeared crucially important to be studied when addressing toxicity. We
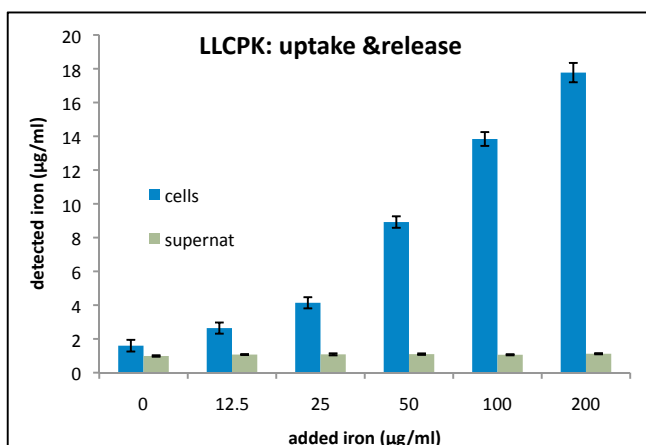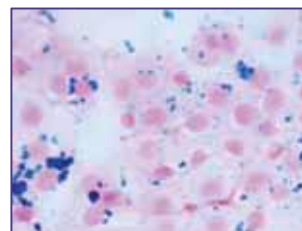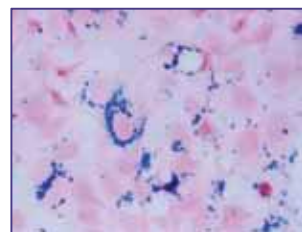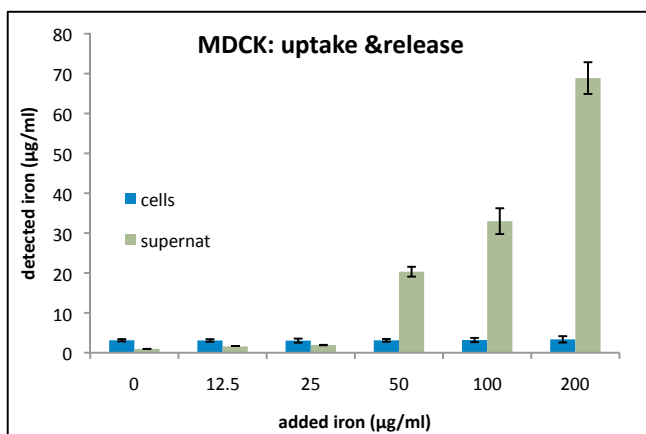
Figure 1. Uptake and release of uncoated iron oxide NPs by MDCK and LL-CPK kidney-derived cells. The cells were exposed to NPs for 24h, then fresh culture medium without NPs was added for another 24 h, and iron levels were measured in the cells and in the supernatants. The blue bars are iron levels in cells left after the 24h release period, so keep up of iron NPs by the cells. The green bars are the release during 24h of the NPs into the culture medium after their uptake by cells. Uptake of uncoated iron oxide NPs was found to be time-concentration and cell line-dependent. Release from MDCK cells, but not from LL-CPK cells.

completed this year all uptake studies and selected the best methods to be suggested in battery of assay.

In Vitro Screening Tests: Oxidative Stress

• Oxidative stress was measured in different models. With the exception of PLGA PEO NPs, all NPs tested demonstrated some level of induction of oxidative stress. The biotic production of ROS was evaluated using fluorescent probes dihydroethidium and DCFH-DA and thiol depletion by monobromobimane.

All NPs have been tested on airway epithelial cells (alveolar and bronchial), brain endothelial cells and primary hepatocytes. Production of intracellular ROS in BeWo cells has been assessed after treatment with SiO2 NPs and in human blood granulocytes after exposure to iron oxide NPs.

Progress made over the last year: Oxidative stressed measured with all NPs, SOPs developed (none last year) and selected for recommendation.

• The cytotoxicity of reference NPs was tested in different models. Cytotoxicity in airway epithelial cells depends on the coating, size of the NPs and their metallic nature. All NPs tested demonstrated some level of induction of cytotoxicity at high level of exposure in different cell systems. General toxicity in liver model indicated that toxicity in Kupffer cells was higher than that observed for hepatocytes exposed to the same NPS for the same period of time. Cytotoxicity in lymphocytes exposed to NPs decreases

Figure 2. DNA damage after the treatment of endothelial cells HCEC with reference NPs (uncoated iron oxide and iron oxide coated with oleic, silica 25nm, and TiO2 NPs) measured with the alkaline comet assay. DNA damage was found in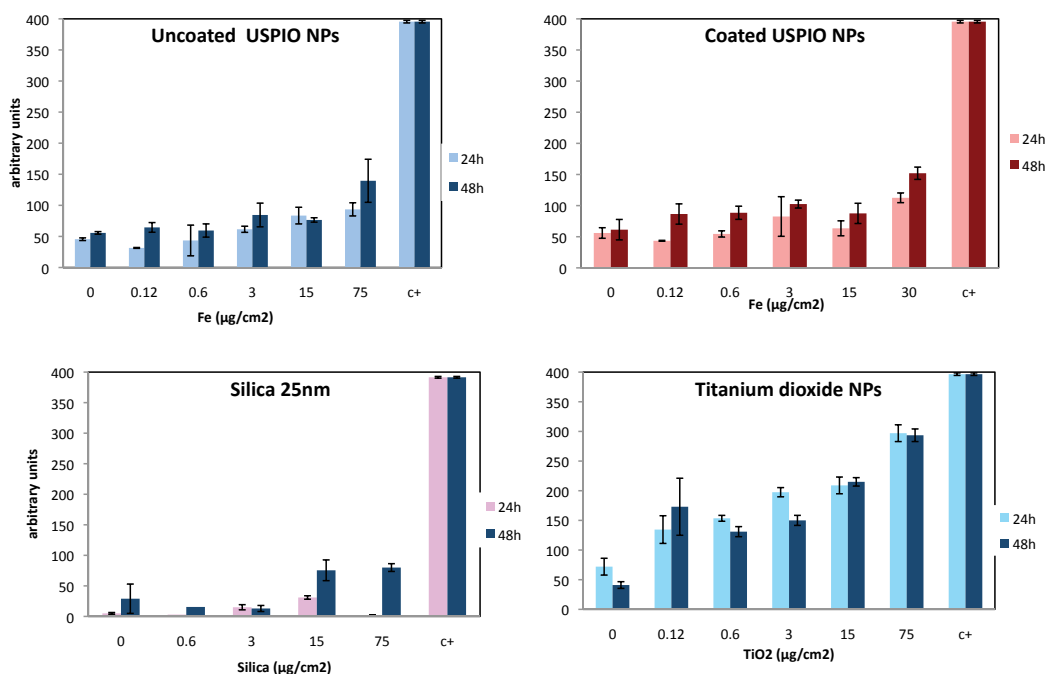 HCEC cells after exposure to iron oxide NPs, silica 25 nm and particularly after TiO2 NPs exposure. No significant DNA damage was found after exposure to PLGA-PEO NPs and Silica 50 nm NPs (data not shown).

in the following order: uncoated magnetite > magnetite coated with oleic acid > PLGA. Silica and TiO2 NPs did not show cytotoxicity to lymphocytes in tested concentrations and time intervals.

Progress made over the last year: Cytotoxicity studies have been measured with all NPs compared with 3 NPs measured last year. Several methods have been selected for recommendation and SOPs have been developed (none reported last year).

In Vitro Screening Tests: Genotoxicity

• Several methods have been employed for genotoxicity testing. The alkaline Comet assay (for detection of strand breaks and alkalilabile sites), modified version of the Comet assay with lesion-specific enzyme formamidopyrimidine glycosylase (FPG ·for detection of oxidised purines), as well as Micronucleus assay. As there was an interference of NPs with cytochalasin B (inhibitor of actin and also endocytosis) the condition for Micronucleus assay used for testing NPs had to be modified and established specifically for this purpose. All reference NPs had been tested for the Comet assay and micronuclei in human blood lymphocytes as well as in lymphoblastoid TK6 cells. Additionally, the Comet assay was performed in cells from all cell organs and tissues apart the gastrointestinal system. We found that the response to

NPs depends on concentration, time of exposure and cell type. The most sensitive cells seem to be endothelial cells. However, the oleic acid coated iron oxide appeared to be most cytotoxic as well as genotoxic, even in non-cytotoxic concentrations, in all cell types.

Progress made over the last year: Genotoxicity (Comet assay and Micronucleus assay) have been completed with all NPs using several cell cultures except with endorem compared with 3 NPs measured with Comet assay and none with micronuclei with limited cell lines last year. Two methods have been modified for nanogenotoxicity testing, SOPs for these tests developed (none reported last year) and selected for recommendation.

In Vitro Screening Tests: Immunotoxicity

• Immune function assays have been used for testing of immunotoxicity potential. Functionality of the lymphocytes was assessed by measuring of proliferative activity after stimulation of the cells with panel of mitogens and antigens. Phagocytic activity and respiratory burst was used to assess function of neutrophils and monocytes. Natural killer cell (NK) activity was analyzed using cytotoxicity assay. Preliminary statistical analysis showed that exposure of lymphocytes to non-cytotoxic concentrations of silica suppressed proliferative response of

cells in cultures *in vitro* stimulated with T- and B-cell mitogens. On the other hand, several examples of stimulatory responses in cultures treated with TiO2, PLGA-PEO and magnetite have been observed. Our results indicate that the most resistant immune response against reference NPs is phagocytic activity of granulocytes. No significant differences were found also in natural killer cell activity of cells treated with non-cytotoxic concentrations of NPs.

Progress made over the last year: Immunotoxicity is completed for most of assays, for most promising methods SOP has been developed (none reported last year).

In Vitro Screening Tests: Assay Automation

• High-throughput screening (HTS) and high-content analysis (HCA) techniques have been applied to develop a set of reliable *in vitro* nanotoxicity assays that can be used for testing large sets of nanomaterials. Candidate assays were selected based on their suitability for automation and the relevance of the toxicological information they provide. Priority was given to endpoints/assays related to oxidative stress and genotoxicity. Manual protocols were translated into automated workflows without compromising any critical steps. The imaging-based HCA assays allow the detection of several biological effects within the same experiment, and have

proven quite tolerant of nanomaterial interferences. Issues regarding reliable dispensing of nanomaterial dispersions have been encountered for some materials but measures are been talking to eliminate this source of variability.

Progress made over the last year: Five assays have been so far selected to be used for HTS and HCA compared with one last year. SOPs have been developed.

In Vivo & Ex Vivo Studies

• *In vivo* toxicity studies have been performed to validate *in vitro* findings. Female rats were exposed to 3 doses of oleic acid coated irone oxide NPs, 0.1%, 1% and 10 % of LD50 (detemined in acute toxicity study according to OECD TG425) by intravenous injection. A range of biomarkers have been followed in parallel to *in vitro* studies to be able to compare *in vivo* with *in vitro*. Markers of cardiotoxicity (mitochondrial coenzyme Q, oxidative phosphorylation), hepatotoxicity, damage in lung tissue cells, renal toxicity, genotoxicity (micronucleus test in bone marrow, strand breaks and oxidised DNA damage in white blood cells and lung tissue cells by the Comet assay), oxidative stress (in liver, lung, brain, heart tissues and in blood), immunotoxicity (immune function assays: proliferation activity of lymphocytes, phagocytic activity and respiratory burst; immunophenotypic

analysis of leukocytes, expression of adhesion molecules on leukocytes, cytokines/chemokines in serum or spleen), routine basic haematological examinations, and histopathology have been measured and are being abalysed.

Progress made over the last year: Considerable progress compared with last year has been made as no experimental *in vivo* work was conducted last year. This reporting period, both acute toxicity as well as main *in vivo* study started and have been completed. Analysis of many biomarkers has been finalised, some endpoints and biomarkers are still being analysed.

- Additionally, small pilot human study was carried out on 12 human volunteers for assessment of possible genotoxic and immunomodulatory effects of the 6 selected NPs in peripheral blood cell cultures. Samples of fresh human peripheral blood of volunteers were exposed to PLGA-PEO, TiO2 (2 dispersions), uncoated and oleic acid coated iron oxide and silica NPs for 4, 24h and 48hs. Results are completed for 10 volunteers. Preliminary data show that there might be individual susceptibility in response to NPs.

Progress made over the last year: Individual susceptibility using immune and genotoxicity markers tested with 12 individuals for all NPs (small population trial) started and was completed within this period).

Structure Activity & PBPK Modelling

- The integrated model, which connects real time exposure concentrations to lung deposition and finally to the effective dose in target organs by inhalation has been developed and was used to estimate internal dose from particulate matter in an office environment. Employing the fluid-particle dynamics model to analyse the behaviour of particles, the effect of gravity on inertial particle deposition in a bend has been studied. Depending on the flow characteristics of the carrier fluid, the deposition of particles could be greatly influenced by gravitational effects regarding both the fraction of the particles that deposit and the sites were the deposited particles are located.

- A theoretical modelling approach has been developed to predict the oxidative stress potential of oxide nanoparticles by looking at the ability of these materials to perturb the intracellular redox state. The model uses reactivity descriptors to build the energy band structure of oxide nanoparticles and predicts their ability to induce an oxidative stress by comparing the redox potentials of relevant intracellular reactions with the oxides' energy structure. Nanoparticles displaying band energy values comparable with redox potentials of antioxidants or radical formation reactions have the ability to cause an oxidative stress and a cytotoxic response *in vitro*. The framework is being developed

to guide the development of rational and efficient screening strategies, in particular those that utilise a battery of *in vitro* assays.

Progress made over the last year: Considerable progress has been made compared with last year (see Publications).

Challenges & Solutions

The project met several challenges due to selection, purchase and consequently characterisation of the NPs. This led to an overall delay in the project, specifically for the fluorescent silica NPs. However, all characteristics of NPs are completed including nanosilica and all *in vitro* work is progressing according to the original plan.

WP3 (*in vivo* experiments) were also delayed due to selection of NPs and long period of negotiation for purchase of Fe_3O_4 NPs with provider and consequently in delay with *in vitro* studies. This delay has resulted in less time to compare *in vitro* data with *in vivo* results. This is crucial and unique part of the project towards selection of most reliable and relevant endpoints and biomarkers and for developing testing strategies is considered to be the biggest challenge. As a solution to this, NanoTEST project would benefit from extension period. This will allow us to perform all statistical analysis and compare *in vitro* with *in vivo* as well as human data thus to validate *in vitro* data. NanoTEST is first project focussing on alternative tests for nanomaterial testing.

The consortium generated an enormous amount of valuable results, both in *in vitro* as well as corresponding assays and markers are being studied *in vivo*. This knowledge will be utilised in suggesting most reliable battery of assays and proposing alternative testing strategy for nanoparticle testing.

NPs do not behave as standard chemicals, and several of the *in vitro* assays have been challenged with some type of interference of NPs, either with detection systems or directly with methods. We collected lots of data to address the technical problems with methods that need to be considered when testing nanomaterial. These results are included into the draft 'intereference manuscript' where we address all critical aspects of NPs testing.

Next Steps

In 2011 our focus will be to complete all the experimental work, finalise missing SOPs and to analyse and compare data from different cell types, NPs, endpoints, as well as assays. Most of the *in vitro* data are finished. It will therefore be crucially important to upload all data to database for statistical analysis and comparison across cell line, endpoints, NPs as well as *in vitro* versus *in vivo*. Based on the knowledge achieved from the experimental data, we will be able to select the most promising assays and for suggesting testing strategy.

Five assays have been so far been selected for HTS and HCA screening and during

2011 the automated protocols will be further optimised and validated. The aim is to test a set of over 20 nanomaterials, including the NanoTEST materials plus selected materials from the nanomaterials repository at the European Commission Joint Research Centre.

The *in vivo* work is in progress and will be finalised this year. We aim to compare the same biomarkers and endpoints investigated in both *in vitro* as well as *in vivo* systems such as immunotoxicity, genotoxicity, markers of oxidative stress, as well as results from *in vitro* histopathology with corresponding results from *in vitro* cell lines or primary cells. This is an important step to evaluate which *in vitro* assays and toxicity data corresponds to *in vivo* finding. Thus we will finally be able to contribute to reduction of use of animals in toxicity testing.

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OpenTox
Promotion, Development, Acceptance & Implementation of QSARs for Toxicology

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Duration: 36 months
Website: opentox.org

Background

OpenTox provides tools for the integration and management of data from various sources (public and confidential) and for the generation and validation of (quantitative) structure-activity relationship ((Q)SAR) models, and their reporting. The design of the OpenTox framework has been guided by user requirements, OECD validation principles, REACH regulatory guidance requirements for *in silico* models, and best practices in computational engineering and open standards. OpenTox supports the creation of applications for toxicological experts without *in silico* modelling speciality expertise, as well as interfaces for model and algorithm developers to incorporate new components and to develop new applications. OpenTox moves beyond existing attempts to create individual research resources and stand-alone tools by providing a flexible, open and standards-based platform that enables the integration of existing solutions and new developments to create a broad and growing suite of applications supporting integrated data analysis approaches in predictive toxicology.

Objectives

The overall goal of OpenTox is to develop an interoperable, extensible modern computing platform supporting interdisciplinary predictive toxicology researcher needs in data management and integration, ontology, model building, integrated testing, validation and reporting. The OpenTox framework supports the integration of state-of-the art *in silico*, cheminformatics, bioinformatics, statistical and data-mining tools, in addition to the automated data management and integration of *in vitro* and *in vivo* toxicology data. The computing components, standards and web services developed by OpenTox enable

the capacity for developers to create a variety of applications satisfying user needs in predictive toxicology.

Experimental Design

Most of the common tasks in toxicity prediction consist of several typical steps, such as access to datasets, algorithms, descriptor calculation, model building and training, and validation procedures. Usually the components that implement these steps are developed from scratch for every new predictive application, and this often leads to undesirable duplication of effort, slow and costly development, and lack of interoperability. The availability of a universal set of interoperable components can facilitate the implementation of new specialised applications that combine algorithms in the most appropriate way and allow fast and rigorous model development and testing. The design objectives of OpenTox were to build a component-based system, independent of programming languages and operating systems, where the components could interoperate between themselves and with external software packages, being able to aggregate data from different sources and staying open for future extensions. OpenTox made two major technological choices in order to keep the developments within these constraints: (i) the 'representational state transfer' (REST) software architecture style, allowing platform and programming language independence and facilitating the implementation of new data and processing components; and (ii) a formally defined common information model, based on the W3C

'resource description framework' (RDF) and communication through well-defined 'application programming interfaces' (APIs) ensuring interoperability of the web components. The OpenTox framework integrates both technologies into a distributed semantic web of web services, where both data and processing resources are described by ontologies—either pre-existing or developed within the project. The framework consists of individual modules, developed by different partners, with different programming languages, running on a set of geographically-dispersed machines and communicating via the internet. The modules can be used to build more complex applications, to embed OpenTox web services into workflows, to build web 'mashups', and to consume web services via client-based software applications.

OpenTox actively supports public standards for data representation, interfaces, vocabularies and ontologies, and open-source approaches to core platform components. The OpenTox Framework currently includes, with its initial APIs, services for compounds, datasets, features, algorithms, models, ontologies, tasks, validation, and reporting which may be combined into multiple applications satisfying a variety of different user needs. The OpenTox approach to ontology allows for efficient mapping of similar and/or complementary data coming from different datasets into a unifying structure having a shared terminology and meaning.

Results

The OpenTox Framework

The OpenTox repository and website *opentox.org* are used for partner and community communications, as well as documentation of all collaborative technical developments on OpenTox. Descriptions of current standards, existing software components and APIs that are relevant for OpenTox applications are made available on the OpenTox website. During the project, the APIs have undergone substantial improvements so as to create semantically-defined interfaces for all OpenTox software components and are published publically on the OpenTox website. These specifications were used as the basis for the development of initial prototype applications.

Data Infrastructure

The OpenTox data infrastructure implemented in Ambit *(http://apps. ideaconsult.net:8080/ambit2)* currently contains quality-labelled data for 185,108 chemicals, and includes ECHA's list of pre-registered substances for REACH along with high-quality data from ISSCAN, JRC PRS, EPA DSSTox, ECETOC skin irritation, LLNA skin sensitisation, and the BCF Gold Standard Database. Additional data for chemical structures has been collected from various public sources (e.g., Chemical Identifier Resolver, ChemIDplus, PubChem) and further checked manually by experts.

Ontology

Interoperability of resources within OpenTox is achieved using a common information model, based on OWL-DL ontology and related ontologies, describing predictive algorithms, models and toxicity data. As toxicological data may come from different, heterogeneous sources, a standardised and deployed ontology is critical for the rational and reliable organisation of the data, and its automatic processing. Up to now the following related ontologies have been developed for OpenTox:

- Toxicological ontology, describing the toxicological endpoints
- Organ system ontology addressing targets/examinations and organs observed in *in vivo* studies
- ToxML ontology, representing semi-automatic conversion of the ToxML schema
- ToxLink, including ToxCast assay ontology
- OpenTox ontology, which is a representation of OpenTox framework components – chemical compounds, datasets, algorithms, models and validation web services
- Algorithms Ontology, including types of algorithms.

Besides being defined in an ontology, OpenTox components are made available through standardised REST web services, where every compound, data set or predictive method has a unique resolvable 'unique resource identifier' (URI), used to retrieve its RDF representation, or to

initiate the associated calculations and generate new RDF resources with their own URIs.

OpenToxipedia.org is a new related resource of toxicology terminology organised in Semantic Media Wiki format. OpenToxipedia allows creation, addition, editing and storing terms used in both experimental toxicology and *in silico* toxicology. It is a community knowledge resource. The particular importance of OpenToxipedia lies in the definition of all significant terms used in OpenTox applications such as ToxPredict and ToxCreate.

A Toxicology Ontology Roadmap workshop was jointly organised by OpenTox and the EBI Industry Forum in November 2010; a draft roadmap publication is currently in preparation.

Authorisation & Authentication

The ability to integrate confidential data, well protected from unauthorised access, was implemented into OpenTox by an authentication and authorisation infrastructure. This infrastructure deals with 'who' has the right to do 'what' with the confidential data. It involves confirming the identity of the user who requires access (authentication) and then putting this confirmed identity against a set of restrictions to determine whether the requested access should be granted or denied (authorisation). The restrictions themselves are defined through different access control policies. We implemented OpenSSO—a single sign-on authentication

and authorisation server by Oracle—for all secured OpenTox services. A policy configuration service was developed in order to define and manage the access control policies. Finally, a common user and policy database was established. With this functionality, OpenTox meets a key requirement imposed by the REACH legislation, according to which registrants may ask for protection of the security and confidentiality of the supplied information. We developed a web service approach to validation against confidential data that protects confidential data from unauthorised access during model validation. An alternative implementation that maximises protection of confidential data by providing a local standalone installation of the OpenTox services, not involving any transmission of confidential data over the Internet, was also developed.

Applications

ToxPredict

ToxPredict.org satisfies a common and important situation for a user wishing to evaluate the toxicity of a chemical structure. As illustrated in Figure 1, the user may upload or draw the chemical structure in a web browser and quickly obtain a report back on what current data and model predictions are available for the toxicity endpoints in which they have an interest. The user does not have to cope with many current challenges, such as the difficulty of finding or using existing data or creating and using complicated computer models. Because of the extensible nature of the standardised design of the
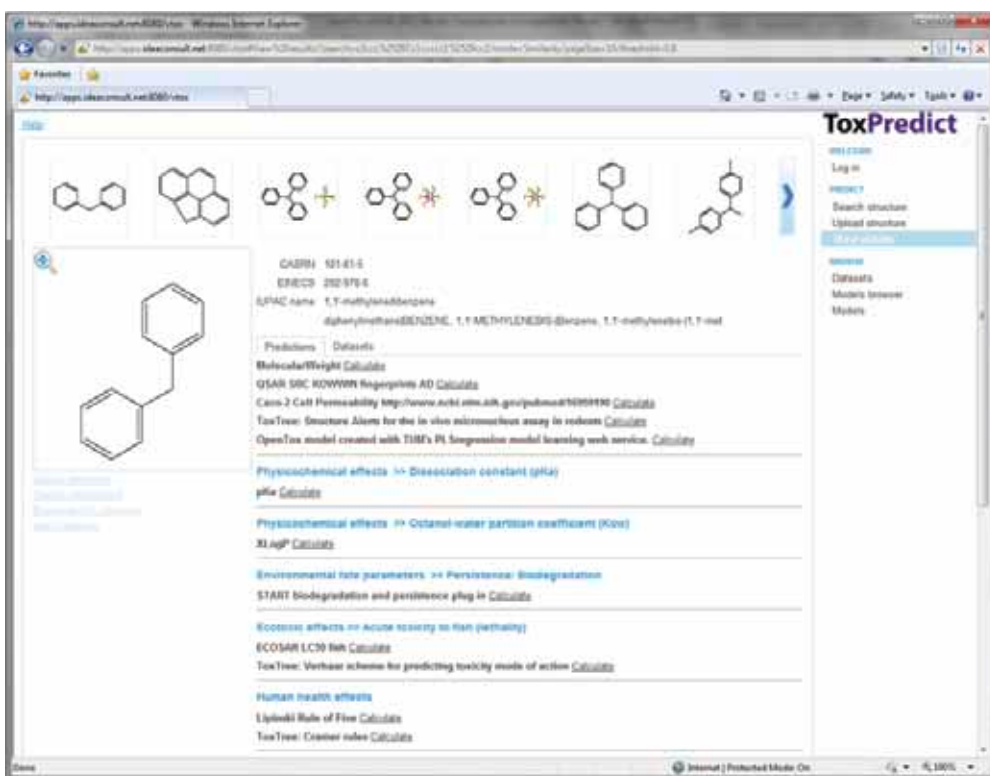
Figure 1. ToxPredict: OpenTox Application for model prediction of toxicity endpoints for chemical structures involving the combination of distributed multiple predictive toxicology resources.

OpenTox framework, many new datasets and models from other researchers may be easily incorporated in the future, both strengthening the value offered to the user and ensuring that research results are not left languishing unused in some isolated resource or stand-alone application not accessible to the user.

Some of the major functionalities of ToxPredict include chemical searching or upload, browsing of available experimental data, performing descriptor calculation and running model predictions. Currently it provides access to 18 ready

to use models, addressing 23 different endpoints and this list is continuously growing. The endpoints include among others carcinogenicity (ToxTree: Benigni/ Bossa rules for carcinogenicity and mutagenicity), eye irritation/corrosion (ToxTree: Eye irritation), skin irritation/ corrosion (ToxTree: Skin irritation), skin sensitisation (ToxTree: skin sensitisation alerts (M. Cronin)), gastrointestinal absorption (OpenTox model created with TUM's kNN regression model learning web service), human health effects (Lipinski Rule of Five, ToxTree: Cramer rules), protein-binding (SmartCYP: Cytochrome

P450-Mediated Drug Metabolism), Caco-2 cell permeability, persistence-biodegradation (START biodegradation and persistence plug-in), acute toxicity to fish (ECOSAR LC$_{50}$ fish, ToxTree: Verhaar scheme for predicting toxicity mode of action). Some additional models predict the dissociation constant (pKa) and the octanol-water partition coefficient (Kow).

ToxCreate

ToxCreate.org provides a resource to modellers to build sound predictive toxicology models basely solely on a user-provided input toxicology dataset that can be uploaded through a web browser (Figure 2). The models can be built and validated in an automated and

Figure 2. ToxCreate: OpenTox Application for the creation and validation of a predictive toxicology model involving the combination of distributed multiple predictive toxicology resources.

scientifically sound manner, so as to ensure that the predictive capabilities and limitations of the models can be examined and understood clearly. Both physicochemical and biological properties can be used as model descriptors. A new web service for the authentication and management of user permissions has
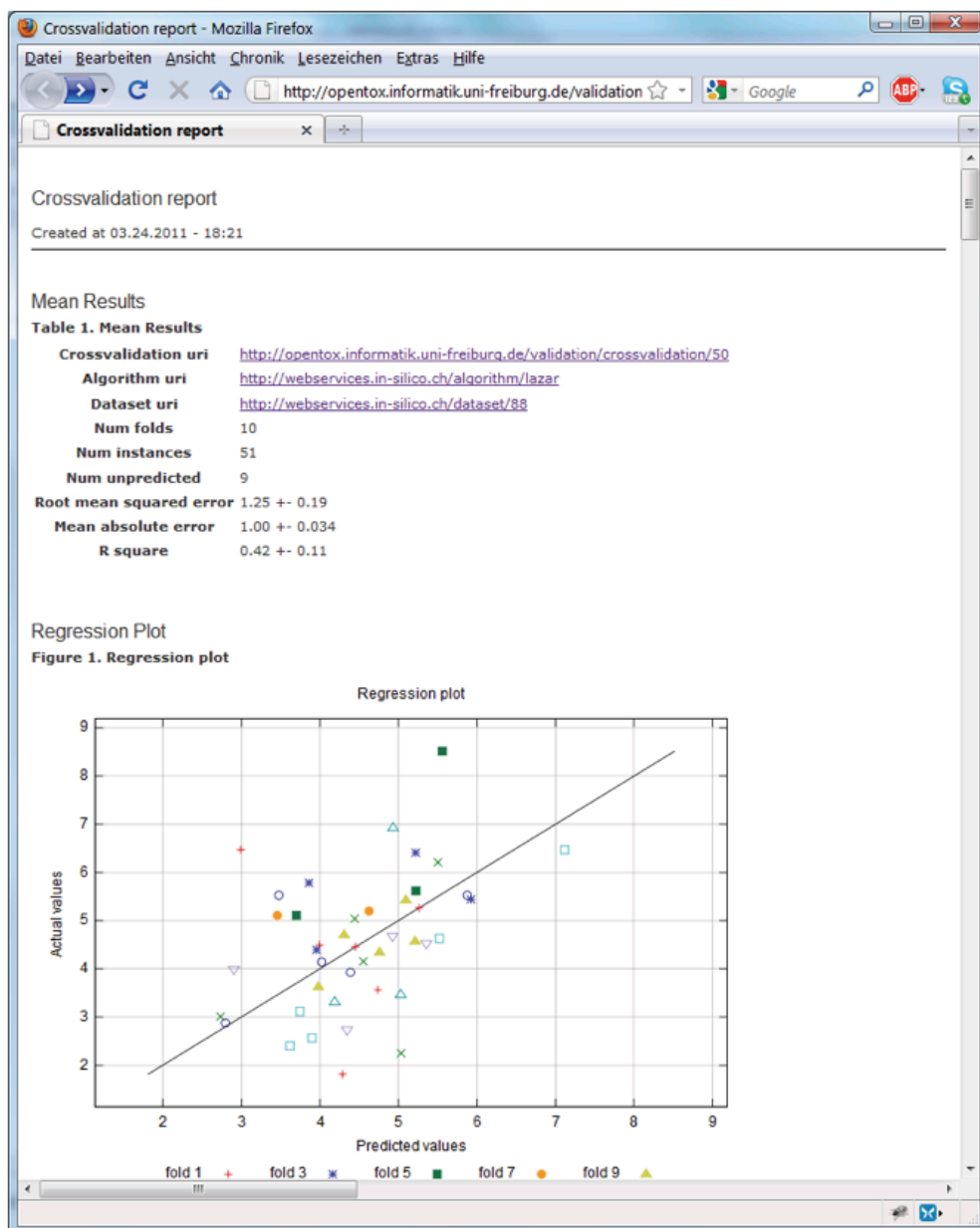
Figure 3. OpenTox validation report for ToxCreate model.

been implemented. With a valid OpenTox account, users can now create and manage models and datasets that are private, i.e., protected from deletion by anyone else. The user interface was extended to include progress bars for model generation, re-nameable data sets, and expandable/collapsible menus. Prediction results of models are accompanied by confidences in predictions. Models can subsequently be made available to other researchers and combined seamlessly into other applications through the OpenTox framework.

Validation

The validation of QSAR models was integrated into the ToxCreate application to provide overview validation statistics as well as a detailed report. A QMRF (i.e., 'QSAR model reporting format' included in the REACH guidance) report is now created for every model in ToxCreate and can easily be edited, using a full QMRF editor initiated as a Java web start application. Whenever a model is created within ToxPredict, it is evaluated via 10-fold cross-validation. The summary of the validation results is provided in the user interface; a more extensive report is available through a link that provides details plots and statistical analysis (Figure 3).

Q-Edit

In order to facilitate considerations on the adequacy of the prediction (model result) in relation to a defined regulatory purpose[1], the European Commission Joint Research Centre[2] has compiled a standard for

reporting (Q)SAR predictions for chemical compounds. The '(Q)SAR prediction reporting format' (QPRF) is a harmonised template for summarising and reporting substance-specific predictions generated by (Q)SAR models and is included in the REACH guidance[3].

Q-edit (*github.com/alphaville/Q-edit/ downloads*) was developed as a QPRF report editor that allows end-users to exploit OpenTox web services to automatically retrieve information needed to create a QPRF report. Users can create a QPRF report, store it in a modifiable form, export it as a PDF, and create password-protected QPRF reports. All these functionalities are orchestrated by the OpenTox web services with which the user doesn't have direct interaction as they interact with a visual interface (Figure 4).

While working through the Q-edit workflow, the user can search online for a compound within OpenTox, enter additional meta-information (e.g., on stereo-chemical features that might affect the validity of the prediction), select and inspect a model including features and algorithms used, find QMRF reports created for that model, use the model to obtain a prediction for the compound, acquire a list of structural analogues of the compound for which the QPRF report is created, and append discussion and export the report in PDF format.

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[1] QPRF report for formaldehyde as an example: http://is.gd/wyLx7D
[2] Joint Research Council. See: http://ec.europa.eu/dgs/jrc/index.cfm
[3] QPRF specification: http://ecb.jrc.it/qsar/qsar-tools/qrf/QPRF_version_1.1.pdf

Figure 4. Creating a QPRF report with Q-edit.

Read-Across

Read-across is a well-known process in human risk assessment. In the REACH guidance it is intended for use in predicting properties of substances without specific testing information for several endpoints including human health hazards (e.g., acute toxicity, skin sensitisation, carcinogenicity, specific target organ toxicity after repeated exposure). We developed a use case for the read across process for repeated dose toxicity for hazard assessment. The N(L) OAEL (no (lowest) adverse observed effect level) can be used to characterise the hazard according to the criteria set up by the GHS (*osha.gov/dsg/hazcom/ghs. html*). The use case implementation was developed using similar approaches as described for the ToxPredict application.

CheS-Mapper

Scientific researchers in the field of chemoinformatics are often overwhelmed by the size and the sheer complexity of chemical datasets. We have developed the 3D molecular viewer CheS-Mapper (Chemical Space Mapper) to enable the visualisation of complex chemical

Figure 5. Visualisation of clustered compounds with CheS-Mapper.

datasets used in predictive toxicology model building. Large datasets are divided into clusters of similar compounds and consequently arranged in 3D space, such that their spatial proximity reflects their chemical similarity (Figure 5). This intuitively provides essential information to the user, while making the dataset more easily accessible and understandable. The different clustering approaches employed in the tool utilise common substructures as well as quantitative chemical descriptors of the compounds. These features can be highlighted within CheS-Mapper, which aids the user to better understand the underlying scientific knowledge. As a final function, the tool can also be used to select and export a specific part of a given dataset for further analysis.

Descriptor Calculations

ToxDesc (*opentox-dev.informatik.tu-muenchen.de:8080/ToxDesc*) was developed by TUM as an application with a graphical user interface for descriptor calculation. Calculating molecular descriptors is a frequent task in the (Q)SAR model development process. ToxDesc is web-based, which makes it accessible anywhere, and enables calculation of arbitrary combinations of descriptors

Figure 6. ToxDesc descriptor calculation application.

Figure 7. MaxTox model-building application.

available as OpenTox REST web services. Using the web interface, the user can select descriptor calculation algorithms from different providers (Figure 6). If a selected algorithm contains sub-descriptors, a list containing all available descriptors will be shown. If there are parameters available to configure the algorithm, a parameter selection panel is shown next to the table. After selecting all descriptors, the user has to enter a valid dataset URI and select one of the available dataset services, before clicking on the 'calculate' button. After that, the user is informed when the results are available. ToxDesc addresses the use case, where the user wants to calculate different descriptors for a set of chemicals. The descriptors can then be used to model an endpoint. The desired set of descriptors can be a combination of physico-chemical, geometrical and sub-structural descriptors. The resulting dataset can be used directly for modelling. The IBMC DESC application (*http://195.178.207.160/Opentox/ IBMCDesc*) was developed by IBMC for the calculation of descriptors used in their models.

MaxTox

MaxTox (Figure 7) attempts to determine the relationship between the toxicity of a molecule and the 'maximum common substructure' (MCSS) it shares with other toxic molecules. MaxTox currently contains

models formed from three different datasets for carcinogenicity, mutagenicity and TD_{50} endpoints. MaxTox also provides a service to generate fingerprints for a given set of compounds using the MCSS approach which can be used for creating prediction models using machine learning algorithms such as 'random forest' and SVM (*opentox2.informatik.uni-freiburg. de:8080/MaxtoxMCSS/findMCSS*).

Bioclipse-OpenTox

In collaboration with Bioclipse developers, the development of a new platform interoperability in computational toxicology was achieved that is able to dynamically discover OpenTox computational services running the latest predictive algorithms and models, while hiding technicalities by reusing a graphics-oriented workbench for the life sciences (*opentox.org/data/blogentries/ public/bioclipseopentoxdownloadfeb2011*). We implemented this platform, integrating OpenTox and the interactive, but scriptable open-source workbench for the life sciences, Bioclipse. At the core of the interoperation lies the use of RDF and related Open Standards. OpenTox uses RDF as a primary exchange format and the RDF query language SPARQL to discover data sets, algorithms and models. Bioclipse recently gained support for these standards, simplifying the interoperation task with OpenTox. The SPARQL query language is used by Bioclipse to dynamically discover descriptor algorithms exposed via OpenTox services, using the OpenTox ontology service's SPARQL endpoint. This way, when a new

descriptor algorithm or model is registered on the OpenTox ontology service, it will automatically be picked up by Bioclipse. Descriptor calculations from OpenTox can be mixed with descriptor calculations local to Bioclipse, or remotely from other computational services. This creates a flexible application for the integration of numerical input for statistical modelling of toxicological endpoints.

We describe here a use case that is currently working in the Bioclipse-OpenTox application (Figure 8). The user first has searched on the web about the toxicological properties associated with a chemical- biological interaction using resources such as Wikipedia and Google. Upon obtaining a representation for a chemical moiety suspected of potential toxicity in SMILES format, the user may input the string into Bioclipse and view the molecule. Clicking on the play button runs both local Bioclipse models and accesses remote distributed OpenTox services and returns toxicity alerts and predictions on the molecule with regards to properties such as carcinogenicity, mutagenicity and metabolism. The predictions can be linked and visualised with chemical features of the molecule displayed in the drawing tool. The user may additionally edit the molecule and rerun the calculations through hitting the play button again. Figure 8 shows a screenshot of this interaction for a query based on the diterpine excoecarin, a proposed active ingredient responsible for the severe toxicity properties of the Tamboti Tree.
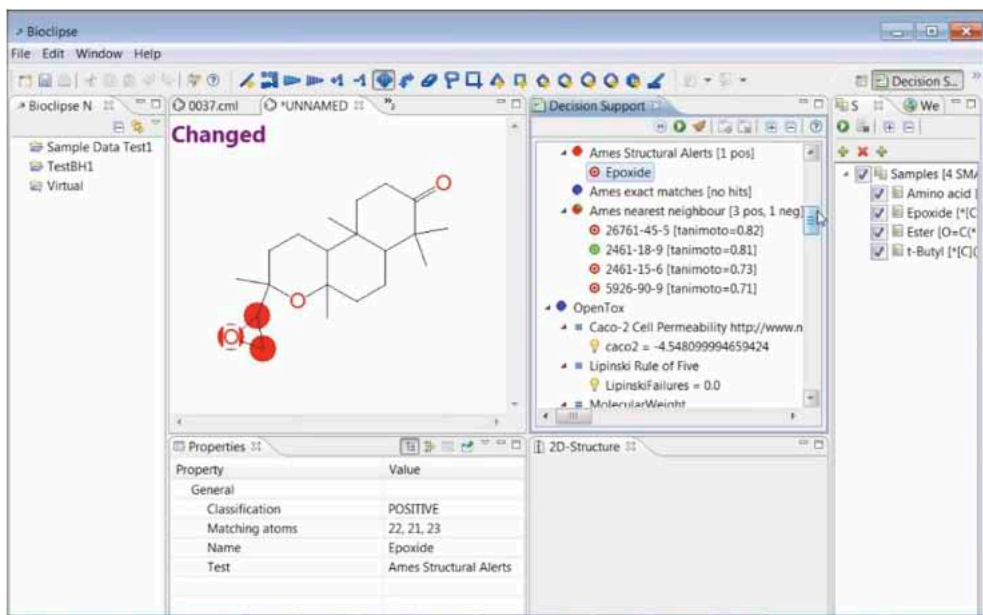
Figure 8. Bioclipse interaction with OpenTox.

Drug Design

In June 2010, the 'Scientists Against Malaria' collaboration (*scientistsagainstmalaria.net*) was formed to apply modern drug design and modelling techniques in combination with industry standard infrastructure and interdisciplinary science to develop new treatments against Malaria. The group's first project assembles a number of leading academic researchers together with smaller innovative companies who are collaborating to develop novel inhibitors active against the *Plasmodium* parasite. The collaborative work is being carried out by a Virtual Organisation (VO) of partners with a combination of scientific competencies and supporting resources. In addition to experimental laboratory and screening facilities, work activities are supported by advanced software systems including a collaborative electronic laboratory notebook, newly developed event-driven collaboration services from the FP7 ICT supported SYNERGY project (*synergy-ist.eu*), and a variety of drug design and modelling software being used by partners. OpenTox is relevant to the drug design activities as toxicity is a major reason for the failure of many drug candidates. Incorporation of OpenTox-based predictive toxicology predictions supports the holistic evaluation of the properties of design libraries to include both activity and toxicity properties.

Workflows

Taverna.org.uk has been used to provide a user friendly workflow system to access and combine OpenTox web services. It provides a user interface which can be

used to generate arbitrary workflows from combinations of single OpenTox web services. The web services are combined by importing single web services and connecting their inputs and outputs using a point-and-click user interface.

Algorithms

During the second year of the project the following algorithms were implemented and made available via OpenTox services:

- Feature calculation: gSpan, CDK (TUM and IDEA), JOELib2 (TUM), FMiner (IST), MOPAC descriptors (IDEA), pKa calculations (IDEA)
- Feature selection: PCA, Chi² feature selection (TUM), Filter feature selection (NTUA)
- Classification and regression: Gaussian process regression, M5' Model Trees, BayesNet, Linear regression (TUM), Support Vector Machine, Applicability domain calculation based on leverage (NTUA), Weka J48 decision trees, Weka MLR, 12 different Toxtree modules (IDEA)
- Additional algorithms: InChI generation (IDEA)
- Superservice for applying models, automatically calculating descriptors.

REACH Models

The following datasets were assembled and used to build QSAR models for REACH-relevant endpoints:

1. Rodent carcinogenicity
2. *In vivo* micronucleus data

3. *Salmonella* mutagenicity
4. Repeated dose toxicity
5. Fish toxicity (fathead minnow)
6. ToxCast endpoints
7. LogP.

The reporting of the results of this model-building exercise and the hands-on application of the models by users took place at the OpenTox workshop in Munich in August 2011 (*opentox.org/meet/opentox2011*).

Tutorials

OpenTox tutorial materials on a number of topics have been prepared and made available online. The topics include walk-throughs of the two end user prototype applications ToxPredict and ToxCreate, illustration of the use of validation and reporting services applied to predictive toxicology models, the application of OpenTox facilities in a drug discovery workflow, and detailed instructions on how to get a system set up to host an OpenTox data service.

The tutorial example on the prototype OpenTox application ToxPredict accepts chemical structures and names as input from the user and generates toxicity reports based on various pre-calibrated toxicity models and existing toxicity data.

In the ToxCreate tutorial, the user provides a dataset of chemical structures and target variable data. ToxCreate subsequently builds and validates a QSAR predictive toxicology model. The user receives a reporting on details of model results

and model predictions, which they may examine, and including using the model for new predictions.

In the *in vitro* data model-building tutorial, a predictive model is built based on *in vitro* data using OpenTox web services. Several models can be built and inspected based on application to the US EPA ToxCast dataset.

The tutorial on web validation and reporting web services, which are also behind the end-user applications ToxPredict and ToxCreate, shows how cURL calls can be used to validate a predictive model or an algorithm. A number of different validation methods are used, including K-fold split, training-test-split and bootstrapping. Furthermore, QMRF reports are generated and visualised using the QMRF Editor web start application.

The objective of the ISSMIC data analysis tutorial is to illustrate searching facilities and data visualisation tools in the OpenTox framework, specifically in the context of *in vivo* micronucleus mutagenicity assays contained within ISSMIC, a curated database, containing critically-selected information on chemical compounds tested with the assay.

A tutorial example of a predictive toxicology application in drug discovery is provided using the data on anti-malarial compounds made available at the ChEMBL Neglected Tropical Disease (NTD) archive (*ebi.ac.uk/chemblntd*). The anti-malarial compounds are prioritised based on a strongly conservative model for predicting oral toxicity. Experimentally-determined cytotoxicities against human cells of the compounds predicted to be safe are further examined, and their mutagenicities predicted. Sites of cytochrome P450 metabolism are predicted for selected compounds with no mutagenicity alerts, low human cytotoxicity, but high anti-malarial activity.

A tutorial is provided to guide the user through the setup of an OpenTox data service based on the download of the Ambit software and its subsequent installation either on Windows or Linux.

All tutorials and their updates are made available on the OpenTox website. An OpenTox workshop was held in September 2010 ahead of the EuroQSAR meeting in Rhodes, Greece and attracted approximately 80 participants who worked through the OpenTox tutorials described above.

Discussion

Current major barriers to progress in the field of alternative methods include a lack of standards, interoperability and coordination between stakeholders resulting in poor integration, stand-alone approaches, high costs and poor sustainability for resources and applications. The applications developed to this date show the potential impact of OpenTox for high-quality and consistent structure-activity relationship modelling of REACH-relevant endpoints. Because of the extensible nature of the standardised Framework design, barriers

of interoperability between applications and content are removed, as the user may combine data, models and validation from multiple sources in a dependable and time-effective way.

OpenTox allows risk assessors to access experimental data, QSAR models and toxicological information from a unified interface. The OpenTox framework includes approaches to data access, schema and management, use of controlled vocabularies and ontologies, web service and communications protocols, selection and integration of algorithms for predictive modelling, validation and reporting, and OpenTox API specifications. The OpenTox framework works independently of any specific algorithm, dataset or endpoint thus providing an extendable capability for the building of many different resources and applications in predictive toxicology.

Our interactions with industry and regulators have revealed that their current requirements in computer-based toxicology relevant to REACH go beyond the QSAR approaches covered by the initial OpenTox description of work, for example: a) integration of QSAR approaches with Weight of Evidence and Integrated Testing Strategies, b) effective combination of *in silico* and new *in vitro* approaches, c) inclusion of knowledge-based approaches, read-across, d) integration of evidence from other model types e.g., from metabolism and pathway models, computational biology, molecular modelling, e) toxicokinetic modelling, f) inclusion of evidence related to mechanism and g) exposure modelling.

We have hence taken such insight into account in the design of the OpenTox framework so that it is a flexible, extensible platform capable of integrating resources, models, predictions and data from both QSAR and other alternative *in silico* and *in vitro* approaches. By openly publishing technical developments including APIs on the OpenTox website, we provide the opportunity for external developers and projects to integrate additional models and algorithms into the framework to satisfy such additional requirements.

Next Steps

We are currently extending the OpenTox platform to include support for a broader range of emerging chemistry and biology research activities so as to support mechanistic studies and modelling, computational chemistry, systems biology and new *in vitro* assay developments.

With the establishment of the OpenTox design, architecture and interfaces now firmly established, we can build and release an increasing number of new applications of relevance to predictive toxicology research, REACH use cases and reporting, and supporting new novel strategies and resource integration. We will leverage the open nature of the platform to collaborate broadly and actively to interoperate with complimentary development initiatives and resources.

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PREDICT-IV

Novel Alternative Testing Strategies for Use in Pharmaceutical Discovery and Development

Contract number: HEALTH-FP5-2007-202222
Project type: Integrated Project (FP7)
EC contribution: € 11,330,907
Starting date: 1 May 2008
Duration: 60 months
Website: predict-iv.toxi.uni-wuerzburg.de

Background

In the development of new pharmaceutical entities, lead compounds are designed on the basis of desired pharmacological effects. Often, many derivatives of these lead compounds are synthesised early in the development for optimised pharmacological response. Toxicity testing of the many compounds with the desired pharmacological effects generated represents one of the bottlenecks in the development of new pharmaceuticals. Toxicity testing is time consuming, requires a high number of demanding *in vitro* and *in vivo* experiments, and large quantities of test compounds. In addition to hazard assessment, the pharmacokinetics of such compounds are still mainly investigated using animals to identify candidate compounds with the pharmacokinetic properties desired.

Toxicity testing usually relies on the identification of certain histopathological changes and clinical parameters and pathology in animals. Toxicity studies range in duration from two weeks to two years and use 5 to 50 animals/dose groups, with usually three dose groups and a vehicle control. From such studies, detailed information on adverse effects and their dose-response is obtained, but the data generated require extrapolation to the human situation. This extrapolation often fails.

The inclusion of biomarkers for undesired effects is not yet performed in many of the routine toxicity studies, but biomarkers to predict toxicity for relevant target organs are under development and are increasingly applied to predict possible toxicities in the early phase of toxicity studies.

In vitro toxicology using isolated or cultured cells mainly focus on the mode-of-action of a chemical on the cellular level to study toxicodynamics. The results are usually integrated as support for the in vivo studies, but such mechanistic studies are also often initiated after results from in vivo studies are available for confirmation of mode-of-action. Some specific in vitro studies are used for pre-screening to exclude specific unwanted effects.

Current animal toxicity testing has a fairly high predictivity for adverse effects. The main causes of failure in drug development are due to toxicity and lack of efficacy. In fact, unpredicted toxicity in animals accounts for 25% and human adverse events account for 11% of development failures, making toxicity/safety the major cause of drug attrition. There are several causes of poor correlation between animal and human toxicities. One of the main reasons is that animal species do not generally predict human metabolism. Also, the diversity of human patients and the different life-style susceptibility factors do not reflect the well controlled experimental animal settings. Therefore, it is crucial to understand why individuals respond differently to drug therapy and to what extent this individual variability in genetics and non-genetic factors is responsible for the observed differences in adverse reactions. In addition, drug withdrawal from the market due to toxicity is the 'worst case' for a pharmaceutical company. Again, the major reason for withdrawal is unpredicted toxicity in humans, mostly of an idiosyncratic nature and with the liver being the predominant

organ affected. The use of human and mammalian cell-based assays plays a key role in this endeavour. Expert knowledge is required to integrate the many potential mechanisms of toxicities into the safety assessment process and to develop useful non animal-based systems to mimic these events in vitro, preferably at the earliest stages of drug development.

Objectives

The PREDICT-IV project aims to expand the drug safety in the early stage of development and late discovery phase. Using an integrated approach of alternative in vitro methods, a better prediction of toxicity of a drug candidate will be developed. By combining analytical chemistry, cell biology, mechanistic toxicology and in silico modelling with new advanced technologies such as 'omics' and high-content imaging, a link between classical in vitro toxicology and modern systems biology will be set. The integration of systems biology into predictive toxicology will lead to an extension of current knowledge in risk assessment and to the development of more predictive in vitro test strategies. This will enable pharmaceutical companies to take the decision on exclusion of drug candidates due to adverse effects well in advance of performing animal safety testing. PREDICT-IV will evaluate the toxicity of the most frequently affected target organs such as kidney and liver, complemented by neurotoxicity assessment using newly developed in vitro neuronal models.

The ultimate goal is to provide an

integrated testing strategy together with sensitive markers of cell stress in order to predict toxicity prior to pre-clinical animal testing. Such integrated *in vitro* strategies will successful result in a reduction of animal experimentation and thereby decreasing the cost and time of lead compound identification.

In accordance with these objectives, PREDICT-IV is structured as a large collaborative integrated project with 7 scientific work packages (WPs) and 20 contributing partners from academia, government bodies, industry and SMEs.

Deliverables & Milestones Achieved During 2010

WP1: Database Facilitating Integrated Research

All collected data were deposited in a data structure (SDRS) that was designed and realised for the PREDICT-IV project. All data are accessible via the internet, and project partners use the platform as a data warehouse as well for consecutively applied data manipulation. This facilitates the identification of early and late biomarkers that should indicate activation of biological pathways for chronic stress and cell dysfunction. Second the results from kinetic measurements are also collected on this platform leading to new insights about *in vivo/in vitro* differences about absorption, metabolism and excretion.

WP2.1: Characterisation of Long-Term Human & Rat Liver Cell Systems

As a result of the optimisation for the rat liver cell systems the 24-well plate format was chosen to deliver reliable cytotoxicity data. Compared to the parallel tested 96-well plate format the instability of the collagen overlay during medium change and cell death after a short period of cultivation was less intensive in the former configuration.

Characterisation of rat hepatocytes showed that they are highly polarised and that their function depends strongly on extracellular contacts within the culture. In serum-free sandwich culture the primary rat hepatocytes restore their cell-cell contacts and the polygonal hepatocyte like cell shape. In addition, isolated primary hepatocytes form bile canaliculi-like structures, which have been shown to have functional activity via fluorescence microscopy. The fluorescence dye Carboxy-DCFDA is transported specifically by Mrp2 out of the cell into the bile canaliculi like structures. Caspase activity of a serum-free sandwich culture of primary rat hepatocytes is low and the intracellular GSH content is close to physiological levels.

Activities and mRNA levels of varies drug metabolising enzymes and transporters was shown to be near the *in vivo* level and stable over time in culture. Basal activities of CYP1A, CYP2B, CYP2C and CYP3A were measured by fluorimetric (EROD-CYP1A, BROD-CYP2B) or luminometric assays

(2C-Glo-CYP2C, 3A-Glo-CYP3A) while the activity of CYP2B was not detectable in the first three days of culture. The inducibility of CYP1A, CYP2B, CYP2C and CYP3A was shown after 14 days.

Testing of selected compounds & characterisation of toxicities (liver models) (due in 2012 but work ongoing throughout 2010

For the dose-finding, primary rat hepatocytes grown in 96-well collagen monolayer were used, whereby ATP concentration was assessed after 24h treatment. Appropriate doses were then chosen for a second cytotoxicity screen using 24-well plates with cells in the collagen sandwich format. Three concentrations around the mean TC_{10} of every compound were tested again with one biological replicate in a 6-well Roche collagen sandwich culture with daily dosing and an ATP test after 14 days. This was performed to confirm the correct choice of doses for the main -omics/kinetics studies, which was to be performed in 6-well plates.

All 11 compounds for hepatotoxicity models were internally classified/divided into two subgroups, namely (i) non-kinetic (Acetaminophen, EMD 335825, Fenofibrate, Metformin, Troglitazone, Rosiglitazone, Valproic acid) and (ii) kinetic substances (Amiodarone, Chlorpromazine, Cyclosporine, Ibuprofen), which will be processed separately. A total of three or four biological replicates for the non-kinetic compounds group were finalised and all 'omics samples sent to the corresponding partners of WP4.1. Transcriptomics data were generated for these 7 compounds and preliminary data interpretation is available. More detailed analysis, including compound specific mechanistic insights, is ongoing.

Primary Human Hepatocytes

The human-derived hepatocarcinoma HepaRG cell line exhibits limited karyotypic alterations and shows the property of transdifferentiation. When seeded at low-density (LD cultures) they rapidly recover markers of hepatic bipotent progenitors and at confluence they differentiate into hepatocyte-like and biliary duct-like cells (around 50% of each). Maximum differentiation is attained after two-weeks exposure to 2% dimethylsulfoxide (DMSO). By contrast when seeded at high-density differentiated cells retain their differentiated status (HD cultures).

The following major observations have been made:

• In both LD- and HD-seeded culture conditions HepaRG cells retain their differentiated state at both the mRNA and activity levels. No significant changes were observed for most functions studied. A number of genes have been analysed at the mRNA levels: 10 CYPs (CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4), two phase II enzymes [glutathione transferase A1/A2 (GSTA1/A2) and UDPglucuronosyl transferase 1A1 (UGT1A1)], five

membrane transporters [breast cancer resistance protein (BCRP), bile salt export pump (BSEP), multidrug resistance protein-1(MDR1), multidrug resistance-associated protein-2 (MRP2), and Na+-dependent taurocholic cotransporting polypeptide (NTCP)] and three nuclear receptors [aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), and pregnane X receptor (PXR)]. Nine CYP activities have been measured using specific substrates and two plasma transporters using fluorescent probes.

- Moreover, in both seeded conditions HepaRG remain responsive to prototypical inducers (phenobarbital, rifampicin, omeprazole). The fold-inductions are usually much higher than in conventional primary human hepatocyte cultures.
- No significant differences were observed by using HepaRG cells between passages 12 and 20.
- For a number of functions related to drug metabolism their levels are DMSO- dependent. Maximum levels are maintained in cells cultured in the presence of 2% DMSO.
- The cells can be maintained for a few days in serum-free medium without DMSO with the capability of remaining fully responsive to prototypical inducers.

WP2.2

Experiments using untreated human renal epithelial cells (RPTEC/TERT1 cells) under static and perfusion conditions with and without hypoxia have been carried out and submitted for -omic analysis. The experiences and results have provided a basis for developing optimal conditions for cell culture treatment and optimal protocols for cell harvesting.

From 25 nephrotoxins tested, 15 suitable compounds were used to generate IC_{10} values in 96-well plates of differentiated RPTEC/TERT1 cells treated daily for two weeks. Several independent parameters were used to assess toxicity including resazurin, ATP and lactate production. High ($\sim IC_{10}$) and low (NOEL) values were selected and experiments were repeated using differentiated cells cultured on micropours supports. From this a priority list of 8 compounds was selected for further experimentation.

All 8 compounds, 2 including a hypoxia component, have been tested in long-term repeat dose exposures on micro-porous supports at two concentrations and submitted for transcriptomic analysis

WP2.3: Assessment of Drug-Induced Toxicity Based on Electrical Activity Measurements in Cortical Primary Culture (2D), Assessment in Re-aggregating Brain Cultures (3D) of Drug-Induced Changes in Cell Type-Specific Gene Expression & Enzymatic Activities & Evaluation of the Sensitivity of Mechanistic Endpoints

After selection of compounds relevant for CNS toxicity dose range finding experiments were performed using both

neuronal models 2D (primary culture of mouse frontal cortex) and 3D (re-aggregating rat brain). The initial range of tested concentrations was established by cytotoxicity assay based on the results obtained from the *in vitro* blood brain barrier (BBB) permeability studies. Using *in vitro* BBB model (co-culture of brain capillary endothelial cells and glial cells) permeability of 16 studied drugs was classified as low, moderate or high. Additionally the obtained results will serve as classifiers for future rate determination of compound penetration and will be integrated in a PBK model. The obtained results (both from BBB and cytotoxicity studies) made it possible to define the non-cytotoxic concentration that will be further studied by neuronal and glial specific endpoints using neuronal models.

In the last 12 months dose range-finding experiments were completed and the long-term experiments were conducted with repeated exposure. In conjunction with WP4, WP2 has tested and optimised protocols for each of the 'omic technologies. Further a perfusion culture device, which may serve as an automated dosing system, was tested with the renal cells. It was demonstrated that hypoxia increase toxicity to several nephrotoxins in renal epithelial cells.

The long-term studies were started with all selected cell culture systems and chosen compounds. Sample generation in WP2 was performed in parallel to ensure a consistent use in WP4. Subsequent proteomic, transcriptomic and metabonomic data are generated from a single experiment.

WP2.4: Generation of Static Culture Derived EC$_{50}$s from Selected Compounds Using Primarily Conventional Cytomic Assays in Non-Target Organ Culture Models

In order to establish whether compounds selected are selectively toxic to the organ systems, 4 renal compounds, 3 liver compounds and 1 CNS compound were tested over a dose range for 14 days in RPTEC/TERT cells, 2 fibroblast cell line and in HepaRG cells. Analysis of the data is ongoing. A major outcome was that toxicity profiles were not cell type specific and that supernatant lactate was a very sensitive indicator for cell stress.

WP2.5: Optimisation of Bioactivation Co-Culture Models & SOPs

Work is continuing in the development of co-culture models for bioactivation.

WP3 DLs: SOPs for Selected Analytical Procedures, Reports on Characterisation of a Second Cell System Used in WP2 & Report on the Additional Data on Selected Drug/Model to be Transferred to WP5

Further standard strategies for *in vitro* exposure were elaborated and subsequent tiered routes to measure/estimate them determined. The following parameters have crucial impact on the actual dose that reaches the target:

- Stability of the compound over time
- Adsorption to physical components
- Binding to medium macromolecules, essential proteins
- Free vs. bound concentration over time
- Interaction with cell components
- Metabolic competence.

Other biokinetic processes that are essentially related to absorption, metabolism and excretion, which have often been evoked to explain *in vivo/in vitro* differences, were defined:

- Transport across the cell membranes
- Metabolic competence.

In addition to the specificity of the used cell culture systems, parameters that are essential for each test (drug) item have to be taken into account and controlled:

- Transport mechanism across cell membranes of the test chemical
- Biotransformation pathways.

Major results of WP3 were the implementation of the analytical methods for the compounds Ciclosporin, Ibuprofen, Chlorpromazine and Diazepam. Further several methods for Metformin and Amiodarone were tested. The outcomes of WP3 indicate the use of human hepatocytes in suspension as an alternative for monolayer cultures. The obtained data indicate that suspensions of human hepatocytes can be used for preliminary assessment of kinetic time-points for test compounds with unknown intrinsic clearance or for cytotoxicity tests.

Further studies included the transport of chlorpromazine across the Caco-2 cell membrane. Adjustment of the culture conditions to long-term measurement and endpoint assessment of tight junction integrity was performed. The characterisation of the involved cell lines was continued by testing of the metabolic competence of aggregating brain cell cultures, showing that CYP2B1 and CYP1A1 are expressed in this cell system.

WP4.1: Processed -Omics Profiles Derived from Centrally Managed Raw Data

After finishing the experimental setup for toxicogenomics, proteomics and metabonomics (NMR, MS), the measurement of control samples started. Followed by the analysis of the first long-term samples generated by WP2. This study with Ciclosporin in RPTEC cells was conducted and preliminary data were generated. Those are consistent with the expected pharmacologic and toxicologic effects of Ciclosporin.

WP4.2: Setup of HCI Endpoints for Basic Mechanisms of Cytotoxicity & Apoptosis in Selected Cell Models

The following mechanism-based endpoints and markers for cytotoxicity or apoptosis were set-up in the rat hepatocyte collagen-matrigel sandwich cultures and in the renal RPTEC-TERT1 cell line: Acute and chronic-like toxicity were investigated by exposing rat hepatocytes to cyclosporin A, amiodarone or rosiglitazone at four different concentrations for 1, 3, 7,

10 and 14 days. The liver-specific functional endpoints canalicular transport, intracellular neutral lipids and phospholipids were measured by HCI.

RPTEC-TERT1 cells were subjected to chronic-like treatment with the reference compounds cisplatin, cyclosporin A, zoledronate and vancomycin. Markers of general cytotoxicity (cell number, plasma membrane permeability, nuclear size and lysosomal mass) and biomarkers of kidney injury were evaluated by Cellomics Array Scan. The gold-standard biomarkers, suggested by the Predictive Safety Consortium, clusterin, kim-1 and lipocalin were measured in addition.

WP5

Partners continued to improve the SimCyp simulator with a parameter estimation module and extension of the 'advanced dissolution, absorption and metabolism' (ADAM) model. Dose-response modelling was developed for hepatotoxicity. Methods for functional interpretation of transcriptomics data were also developed. The OmicsNet graph software was used to investigate divergence between transcriptomics data from *in vitro* systems and human *in vivo* samples. Preliminary results have been obtained on hepatotoxicity modelling for dose range finding. The results of PBPK modelling for ciclosporin and diazepam have been published.

Challenges & Solutions

The generation of a comprehensive SOP for the sample preparation for kinetic analysis required a thorough information exchange among the involved partners. Since a sample preparation for optimal kinetic analysis was endeavoured, a compromise for sample processing had to be made. The solution included specific sample preparation procedures due to different cell culture model systems.

Next Steps

- Finishing the experiments with the selected compounds and cell models
- Generation of the corresponding metabonomic, transcriptomic and proteomic data
- Finalising kinetic studies
- Identification of early and late biomarkers of cell stress and dysfunction
- Correlating *in vivo* predictions to *in vivo* observations
- Predicting *in vivo* pharmacokinetics of a drug based upon *in vitro / in silico* data.

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Sens-it-iv

Novel Testing Strategies for *In Vitro* Assessment of Allergens

Contract number: LSHB-CT-2005-018681
Project type: Integrated Project
EC contribution: € 10,999,700
Starting date: 1 October 2005
Duration: 66 months
Website: sens-it-iv.eu

Background

With some forms of allergy on the increase, massive resources are invested worldwide to investigate which compounds are the culprits and why otherwise harmless compounds elicit adverse immune responses. In Europe, the European Commission has been an important investor in research into allergies through Framework Programmes.

To date the identification and evaluation of unknown sensitisers completely relies on animal testing as no validated alternative exists. However, the additional testing of chemicals for allergenicity required by the new EU legislation on chemicals (REACH) is expected to consume millions of animals per year. Conversely, several EU legislations call for significant reductions or even a complete ban on animal testing (i.e., for cosmetics and cosmetic ingredients since March 2009).

Therefore, 31 groups from academia and industry, as well as special interest organisations, have joined within the frame of the Sens-it-iv consortium to develop non-animal tests and testing strategies to assess allergenic potential. This is seen in relation with the use of safe ingredients by the chemical, cosmetic and pharmaceutical industries.

Objectives

A. Scientific

1. Use of functional genomics, proteomics, and immunohistology before and after chemical challenge of tissue slices to address *ex vivo* phenotypic characteristic changes of human epithelial cells (EC), antigen presenting dendritic cells (DC), and effector T-cells.
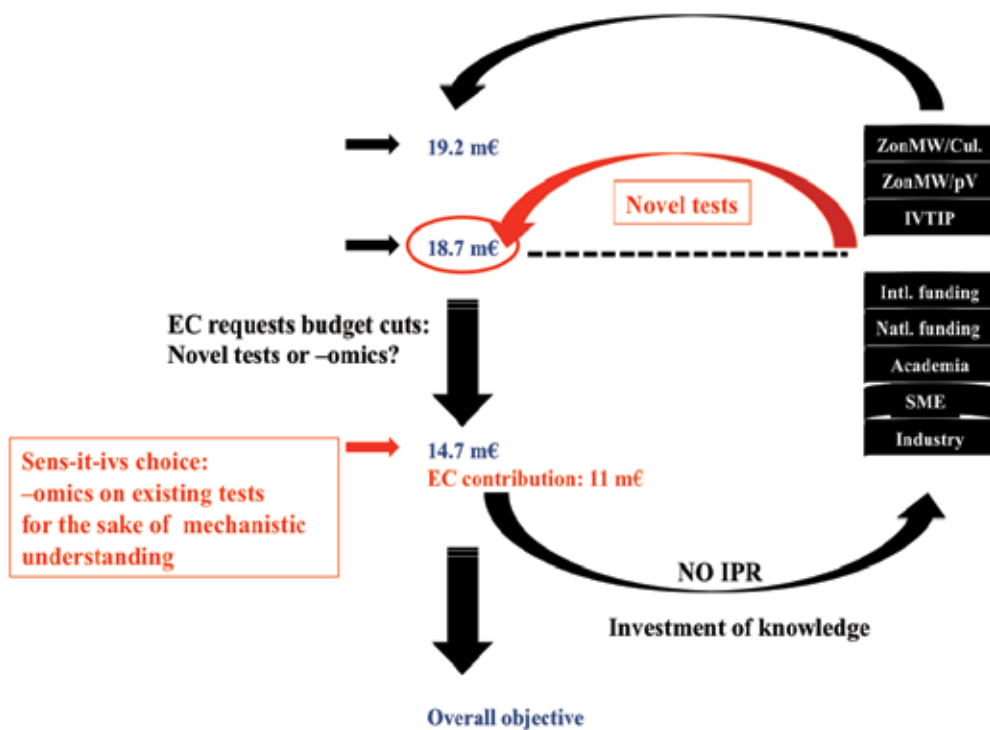
Figure 1. The Sens-it-iv sphere.

2. Establishment of *in vitro* conditions supporting an *in vivo*-like cross-talk between EC, DC and T-cells, and the cascade of cellular and molecular events triggered in such a complex system by a test-compound.

3. Description of the chemical features related to the intrinsic stability of allergens in relation to the metabolic capacity of cells in the target tissue. Chemical structures and peptide sequences involved in hapten-formation are being characterised. The processes of bio-activation and hapten formation are studied by advanced metabonomic and proteomic technology.

B. Technological

1. Setup of an inductive Database for the acquired scientific data, including all available literature information to allow queries for data patterns and predictive models.

2. Development of prototypes of cell-based predictive assays developed by implementation of the cellular and excreted markers suggested by bio-informatics to represent key mechanisms of sensitisation.

3. Refinement and optimisation of these assays for pre-validation.

The ultimate goal is the establishment of *in vitro* assays ready for pre-validation and

approval by ECVAM, the European Center for Validation of Alternative Methods.

Experimental Design

The project that originally was submitted to the European Commission (EC) was budgeted for € 18.7 million. The request by the EC to reduce this budget to €14.7 million forced the Consortium to chose between 'developing novel tests but using existing markers' and 'subjecting existing tests to extensive ·omics analysis'. Considering the ultimate deliverables of the project, the Sens·it·iv choice was to select the second possibility (·omics) for the sake of mechanistic understanding (Figure 1).

During the course of the project, emerging knowledge was invested in an effort to acquire access to the novel tests that were cut out of the budget. This was made possible by the Consortium Agreement making Sens·it·iv an intellectual property rights (IPR)-free project as far as test development is concerned.

This investment was supported by partners from industry and academia, and resulted in additional national and international funding allowing the original objectives related to the development of novel tests to be pursued as well (€ 18.7 million). It has to be stressed that the novel tests coming into the project this way are not the property of Sens·it·iv and therefore do not fall under the Consortium Agreement vis-à-vis IPR. The owners of the tests made the tests available for further development and refinement (WP8), and

marker identification (WP2). Examples of such novel tests include the dendritic cell (DC) migration test, the alveolar-endothelial lung model and the bronchial lung model.

An additional budget (€500,000) was assigned by the Dutch funding agency ZonMW to run a pre-validation study on a selected test approach and to transfer lung EC-based tests to the Cultex system (allowing for aerosol/gas exposure of cell cultures). These activities will extend beyond the Sens·it·iv project (31 March 2012).

Project Structure

In order to make it possible to manage such a large and complex project properly, the Sens·it·iv project was split in 3 modules, each with a specific duration and each grouping specific research activities covered by dedicated work packages (WPs).

As compared to the original structure, three important changes were suggested and accepted by the General Assembly in October 2007 and 2008 (Figure 2).

Training for Pre-Validation (Months 24-38)

This training was introduced in order to familiarise the partners with the procedures of pre-validation before tests were selected for evaluation and refinement (Technology Module). Three very simple dendritic cell (DC)-based assays that were selected. The result of this bridging activity was an understanding for the requirements
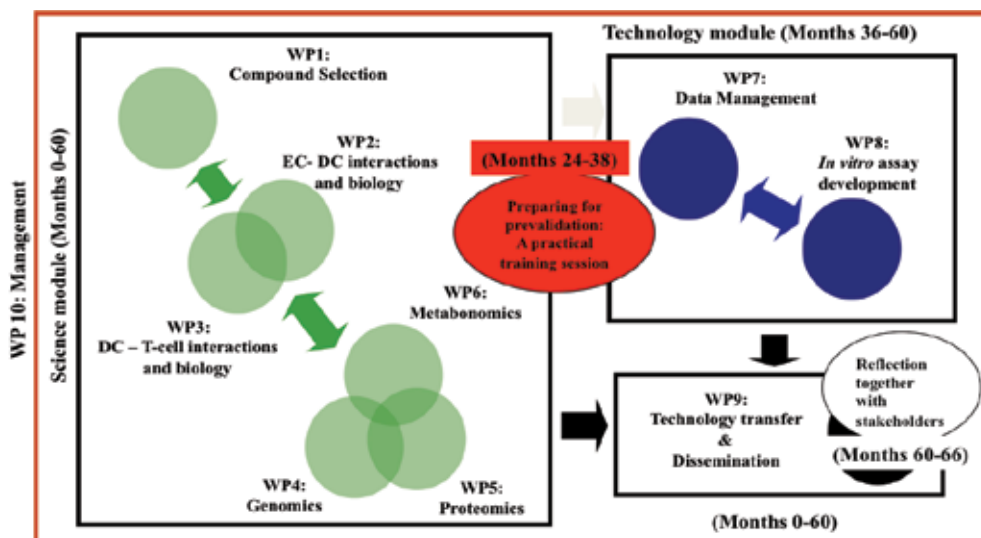
Figure 2. The updated modular structure of the project.

of pre-validation, and focused applied research leading to the tests, which have been under extensive evaluation since the 2008 report. The acquired experience was also implemented on pre-validation project funded by the Dutch funding agency ZonMW.

6-Month Extension (Months 60-66)

This extension is to be used to reflect together with stakeholders (e.g., industry, test developers, regulatory bodies, animal welfare groups) on the outcome of the project. The outcome of these discussions will be published in a peer-reviewed journal.

Extension of Specific R&D Activities

In general, basic research was to be performed during the first 3 years only (2005-2008). However, specific activities

were allowed to continue (Science Module). These activities were selected because further research could give a better understanding of the underlying mechanisms resulting in better tests.

Science Module: Basic Research

WP1 was responsible for the identification of well-characterised test compounds. In this context, 'well-characterised' is to be understood as supported by high-quality *in vivo* data of human origin and from animal experimentation (primarily the local lymph node assay, or 'LLNA').

WP1 was also set to generate and maintain a database collecting information about chemicals and proteins, and tests developed to assess the sensitising potential of these compounds or to study mechanisms of action related to sensitisation and allergy development. This

information has been, and still is, used to assure that Sens·it·iv is not repeating what already was done, but instead is filling out gaps in pre·existing knowledge and is focusing on novel testing systems.

WP2 and WP3 were to develop a detailed understanding of the pivotal roles of epithelial cells (ECs), dendritic cells (DCs) and specific T·cells in the induction of contact sensitisation and respiratory sensitisation. Both WPs have been using a selection of the compounds provided by WP1 for the characterisation of the inter·cellular, intra·cellular and molecular mechanisms that are specifically required for the acquisition of sensitisation. The main difference between the two WPs is that WP2 looks downstream from the ECs toward the T·cells, by using T·cell activation primarily as a tool to evaluate the interaction between ECs and DCs. In contrast, WP3 addresses the issue via the T·cell compartment, by using EC–DC interactions to provide an understanding of how a potential sensitiser may affect these interactions to result in Th2·mediated responses. Since both WPs involve the same cell types, intensive co·ordination and training via WP10 (Management) was provided to assure synergy of the research activities and optimal use of the knowledge acquired.

WP4 (genomics), WP5 (proteomics) and WP6 (metabonomics) provided WP2 and WP3 with the analytical tools for the identification of relevant mechanistic pathways and markers of interest, as well as for the characterisation of cells, cell types and cell·cell interactions.

In addition, WP5 and WP6 were also involved in basic research, contributing to the understanding of hapten·formation and the metabolic activation of chemicals suspected to be sensitisers.

The primarily basic research·oriented activities of WP2, WP3, WP5 and WP6 were stopped by the GA in October 2008, as originally planned in the Technical Annex. However, selected activities were allowed to continue because they had the potential to provide data (i) supporting the relevance of selected tests or (ii) essential for improvement of these tests, or (iii) to identify and explore new opportunities for test development to be pursued by any interested party in the post·Sens·it·iv era.

These specific activities include:

a) WP2:
  • DC migration (until October 2009, review by General Assembly);
  • Interactions between epithelial cells (EC) and DC (until October 2010, review by General Assembly).

b) WP3:
  • Innate immune responses to chemicals (until October 2010, review by General Assembly).

c) WP6:
  • *In vitro* bio·activation (until May 2010, mid·review by General Assembly).

d) WP4 and WP5:
  • Marker identification by a coordinated genomics·proteomics (until May 2010, mid·review by General Assembly).

Technology Module

The aim of technology module was to collect and implement the knowledge acquired by the science module.

WP7 collected from the outset of the project on relevant properly evaluated information from each WP (e.g., standard operational procedures, analytical data, and guidelines) for incorporation into an inductive database. This database was developed into an easily accessible platform for storing raw as well as curated data, running specific queries and sharing information among the partners. Test results collated with other relevant chemo-physical properties entered a programme that was built *ad hoc*, which utilises statistical approaches for a computer-based intelligent testing strategy. In order to optimise this process, a tight collaboration with the FP7 OpenTox project was established.

WP8 involved the application of the stored information for the optimisation and refinement of selected tests (newly developed or existing) up to a level that meets the ECVAM criteria for entering the pre-validation process. Among the tests emerging from the Sens-it-iv project those were selected that had an added value (e.g., improved marker profile, expanded chemical domain) or were novel (e.g., new cell types, cell combinations or mechanisms of action) as judged from the available information.

Communication, Technology Transfer & Dissemination

WP9 has assured visibility of Sens-it-iv by means of a pro-active strategy. The ultimate goal was to ensure early awareness and easier adoption of the *in vitro* test methods by various industry sectors as key end users, academia and regulators. The project website *sens-it-iv.eu* (6,592 unique visitors in 2010) was established and maintained. This website consists of an external site for public dissemination and an internal site facilitating management and communication among the partners. Monthly newsletters summarising in a lay language recent progress were released via the external website and distributed by e-mail (281 subscribers). Sens-it-iv partners COLIPA, ecopa, ECVAM and IVTIP were set to ensure technology transfer and dissemination to the industrial sectors covered by their memberships, to academia, regulators as well as governments, animal welfare organisations and consumers. Technology transfer in the sense of training and education, both internal and external, was provided. Apart from dissemination via regular meetings of stakeholder groups, all the participants were stimulated to provide the proper dissemination of project results through their own channels, including publications in peer-reviewed scientific journals, communications (e.g., e-strategies, The Parliament), oral and poster presentations at inter/national congresses, and via their own networks. With the end of the Sens-it-iv project approaching, WP9 was set to identify and assessing opportunities (e.g., e-learning) assuring that the knowledge

and expertise collected and acquired by the project persists after March 2010.

Project Management

Project management was taken care of by the Co-ordinator, assisted by the Vice Co-ordinator, the Management Team (WP leaders), the Steering Committee, and the Scientific Advisory Board. While these bodies have assured proper and timely exhibition of the implementation plans, or advised on how to proceed (decision making) and to improve, the General Assembly has been the body actually taking the decisions.

An important management task has been to stimulate communication and dissemination among the partners, and to adjust the meeting format to the changing Sens-it-iv landscape. The Science Module was characterised by quarterly progress reports, which were evaluated and discussed by the Management Team. In addition, regular intra-WP meetings were organised (either physical or by telephone) during the first 1.5 years to assure optimal use of resources and to stimulate the exchange of information. As the different WPs started to collect data, integration was stimulated by organising selected inter-WP meetings (e.g., WP2 - WP3 on dendritic cells; WP2 - WP4-6 on –omics analysis of keratinocytes and DC; WP5-6 on haptenisation and bio-activation; WP1-7 on data collection). With the transition from basic research (Science Module) to applied research (Technology Module) the quarterly reports were abandoned. The frequency of GA meetings was increased

from annual to 2 per year since the focus on test development and refinement required an integrated approach and thus the presence of all available expertise. Furthermore, decisions had to be made about (i) tests to be pursued or abandoned, (ii) prioritisation, and (iii) modifications of the implementation plan. Such decisions can only be made by the GA.

Results

Final Compound List

1. Chemicals

The selection of the chemicals to be used for the development of the tests was performed according to predefined criteria.

Inclusion criteria included the following:

- Chemicals should be backed by the availability of high quality *in vivo* data
- Chemicals should be purchasable from commercial sources
- Chemicals should cover as much as possible the dynamic range of effects observed in the *in vivo* tests (e.g., the Local Lymph Node Assay, LLNA)
- Chemicals should cover a relevant range of chemical classes
- Chemicals should cover different chemical reaction mechanisms by which they react with proteins
- Chemicals should cover as much as possible a wide range of physical-chemical properties
- Chemicals that must be activated to react with proteins should be

Table 1: The final compound list.

Respiratory	Skin	Controls
Chemicals (N = 6)	Chemicals – Haptens (N = 20)	Chemicals (N = 20)
Diphenylmethane diisocyanate (MDI)	2,4-dinitrochlorobenzene (DNCB)	Sodium dodecyl sulphate (SDS)
Trimellitic anhydride (TMA)	Glutaraldehyde (GA)	Salicylic acid (SA)
Ammonium hexachloroplatinat (HCPt)	Cinnamaldehyde (CIN)	Phenol (Ph)
Hexamethylene diisocyanate (HDI)	Tetramethyl thiuram disulfide (TMTD)	Glycerol (Gly)
Maleic anhydride (MA)	Resorcinol (Res)	Lactic acid (Lac)
Glutaraldehyde (GA)	Oxazolone (Oxa)	Chlorobenzene (CB)
	Glyoxal (Glx)	P-hydroxybenzoic acid (PHBA)
Proteins (N = 20)	2-bromo-2-(bromomethyl)	
glutaronitrile (BBGN)	Benzaldehyde (BA)	
Dustmite allergen (Der p 1)	2-mercaptobenzothiazole (MBT)	Diethyl phtalate (DEPH)
Phospholipase A 2 (PLA2)	4-nitrobenzylbromide (NBB)	Octanoic acid (OA)
Ovalbumine (Ova)	Formaldehyde (Form)	Zinc sulphate (ZS)
Grasspollen allergen (Phl 5)	Ethylenediamine (ED)	4-aminobenzoic acid (PABA)
Amylase 1 *	2-hydroxyethyl acrylate (HEA)	Methyl salicylate (MS)
Amylase 2 *	Hexylcinnamic aldehyde (HCA)	Ethyl vanillin (EV)
Amylase 3 *	Potassium dichromate (PDC)	Isopropanol (Iso)
Amylase 4 *	Penicillin G (PenG)	Dimethyl formamide (DF)
Cellulase 1 *	MCI/MI	1-butanol (But)
Cellulase 2 *	2-aminophenol (AP)	Potassium permanganate (PPM)
Glycohydrolase 1 *	Geraniol (Ger)	Propylene glycol (PG)
Lipase 1 *	2-nitro-1,4-phenylene-diamine (NPD)	Tween 20 (T20)

Respiratory	Skin	Controls
Protease 1 *		
Protease 2 *	Chemicals – Pre/Pro-haptens (N = 4)	Proteins (N = 1)
Protease 3 *	Isoeugenol (Iso)	Human serum albumin (HSA)
Protease 4 *	Eugenol (Eug)	
Protease 5 *	Cinnamic alcohol (CA)	
Protease 6 *	Paraphenylenediamine (PPD)	
Xylanase 1 *		
Xyloglucanase 1 *		

considered.

Exclusion criteria included the following:

• Gases and highly volatile chemicals
• Insoluble chemicals
• Metals
• High molecular weight chemicals.

With regard to reference data sources for the chemicals selection, two sources were mainly taken into consideration: the LLNA database (Gerberick GF et al., 2005) and the European Centre for Ecotoxicology and Toxicology of Chemicals Technical report N°.77 (ECETOC; 1999) and N°.87 (ECETOC; 2003). [Complete references not supplied]

2. Proteins

The selection of the proteins to be used for the development of the tests has been performed according to predefined criteria.

Inclusion criteria included the following:

1) Proteins should be backed by the availability of high quality *in vivo* data.
2) Proteins should be readily available.
3) Proteins should cover a relevant range of protein classes.

Reference data sources: Protein selection was based upon historical human and animal data from Novozymes AS (industrial enzymes) and the Allergome Platform for Allergen Knowledge (*allergome.org*).

Toolbox

The Sens-it-iv toolbox is the major deliverable of the project and its ultimate legacy after its official termination on 31 March 2011. It contains those assays developed within the Sens-it-iv project, or the Sens-it-iv sphere with contribution of Sens-it-iv, which the consortium found to be worthy of the search for financial support beyond the end of the project.

In contrast to the LLNA, these *in vitro* tests are usually exclusive for skin or

Table 2. The Sens-it-iv Toolbox.

		N	TSF	SOPs	Status
Keratino-cytes	A two-tiered assay combination 1. Measuring intracellular IL-18 levels in a keratino-cyte cell line; 2. Irritation assay in epi-dermal equivalent cultures	30	X	X	PV
Lung EC	Precision cut lung slices	12		X	CT, PV
	Human reconstituted alveolar epithelium	12	X	X	E
	Human reconstituted bronchial epithelium	12	X	X	pv
	Specific sensitiser profile	12		X	pv
DC	Xenobiotic sensing (genomic profile)	49	X	X	pv
	Maturation #2 (DotSCan)	20	X	X	E
	Migration	12	X	X	pv
T-cells	Primary T-cell stimulation	6	X	X	E
Other	Neutrophil - THP-1 metabolism tests	12		X	E
	Proteomics marker profile (combined list)	12		X	E

N: number of compounds tested; TSF: Test submission form (ECVAM); SOP: Standard operation procedure; PV: pre-validation according to the ECVAM guidelines; CT: confirmation test for new markers and marker profiles; E: under evaluation; pv: reproducibility, transferability and reliability assess by Sens-it-iv partners.

lung sensitisers, indicating distinctive mechanisms underlying skin or lung sensitisation. The toolbox does not contain tests that are not novel or do not have added value with respect to test systems and markers developed outside the consortium.

About the Tests

The assay systems of the toolbox include cell types prominently involved in the induction phase of sensitisation such as EC, DC, T cells and neutrophils. All materials, be it tissue, primary cells or established cell lines, are exclusively of human origin, eliminating the problem of reactivity differences between humans and other animals.

In the spirit of the US National Research Council report 'Toxicity Testing in the

21st Century: A Vision and a Strategy' (NRC, 2007), Sens-it-iv emphasised the necessity of physiologically-relevant, metabolic competent and robust assays. Furthermore, -omics based strategies were followed enabling the identification of key pathways of toxicity.

1. Keratinocytes

Most advanced among the tests for skin sensitisation is a two-tiered combination of the keratinocyte assays mentioned in Table 2 and illustrated in Figure 3. Intracellular production of IL-18 by a keratinocyte cell line such as NCTC2544 (tier 1) quantitatively indicates skin sensitisers (including many pro-haptens) but not respiratory sensitisers. The irritation assay (tier 2) of identified skin sensitisers in epidermal equivalent cultures (e.g., CEST1000 (CEllSystems), RHE (SkinEthics) among others) permits their grading according to potency.

The concordance of this two-tiered approach with the LLNA is 79% for potency ranking and 92% for classification into the correct potency groups (extreme, strong, moderate, weak).

2. Lung EC

The assays for respiratory sensitisers include induction of typical activation markers in reconstituted alveolar (developed by Iris Hermanns, Mainz University, Germany) or bronchial epithelium cultures (MucilAir, Epithelix, Switserland) as well as human precision cut lung slices (PCLS) to simulate an *in vivo*-like situation. These tests have been extensively evaluated on both chemicals and proteins, which provided confidence in the capacity of these test systems to identify respiratory sensitisers.

The alveolar and bronchial cell-based tests are presently further assessed
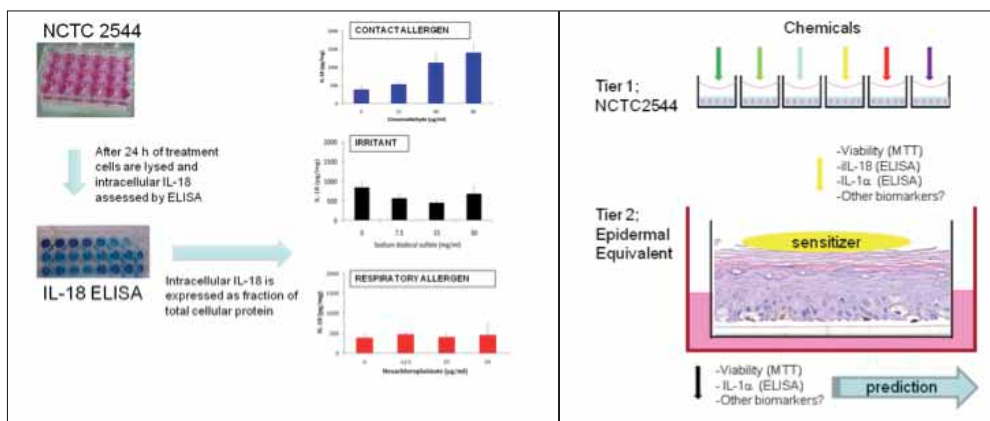

Figure 3. The two-tiered principle.

References (see also Publication section for detailed references): Spiekstra SW, et al. Toxicol In Vitro. 2009; 23, 349-55; Corsini E et al. Toxicol. In Vitro. 2009; 23, 789-96.

Rank	GeneName	Description
1	FAM89A	family with sequence similarity 89, member A
2	STX11	syntaxin 11
3	LIN7B	lin-7 homolog B (C. elegans)
4	RXRA	retinoid X receptor, alpha
6	TGIF1	TGFB-induced factor homeobox 1
8	ERRFI1	ERBB receptor feedback inhibitor 1
10	RNF135	ring finger protein 135
11	MORC2	MORC family CW-type zinc finger 2
12	NETO1	neuropilin (NRP) and tolloid (TLL)-like 1
14	AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9
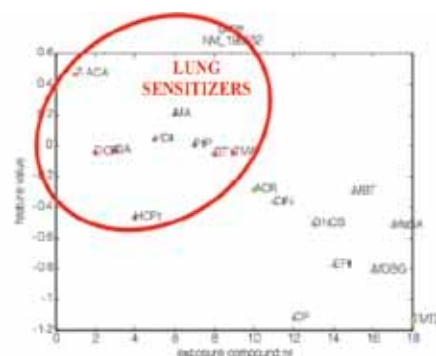
Figure 4. A gene profile for identification of lung sensitisers.

for reproducibility, transferability and reliability by industrial partners in the consumer products and pharmaceutical industries. The intended application is for occupational safety assessment.

With respect to the PCLS, this approach was taken up for pre-validation by the German Government.

Using the human bronchial cell line BEAS 2B, a gene profile of 10 genes was identified, allowing for the identification of lung sensitisers (Figure 4). This profile has successfully been assessed for reproducibility, transferability and reliability within the Sens-it-iv consortium. Currently, no misclassification has been observed with this marker signature.

3. DC-based Tests

3.1. The Genomic Allergen Rapid Detection (GARD) Test for Xenobiotic Sensing

The Genomic Allergen Rapid Detection (GARD) test is a novel test principle for prediction of sensitisers. It is based upon a skin sensitiser-specific gene activation signature (down to 11 genes) in MUTZ-3 DCs. A gene profile for chemical respiratory sensitisers was suggested, but the limited number of chemicals with proven respiratory sensitising potency did not allow yet for assigning any statistical significance to this profile.

The marker signature includes transcripts involved in relevant biological pathways, such as oxidative stress and xenobiotic induced responses, which sheds light on the molecular interactions involved in the process of sensitisation. Besides opening for the possibility to add some of these new markers to existing activation and maturation assays (e.g., HCLAT, MUSST), studies were performed to assess the potential sensitisers by use of a single custom made gene array. The current accuracy of this test in identifying skin sensitisers is 98% with an 11 genes marker profile.
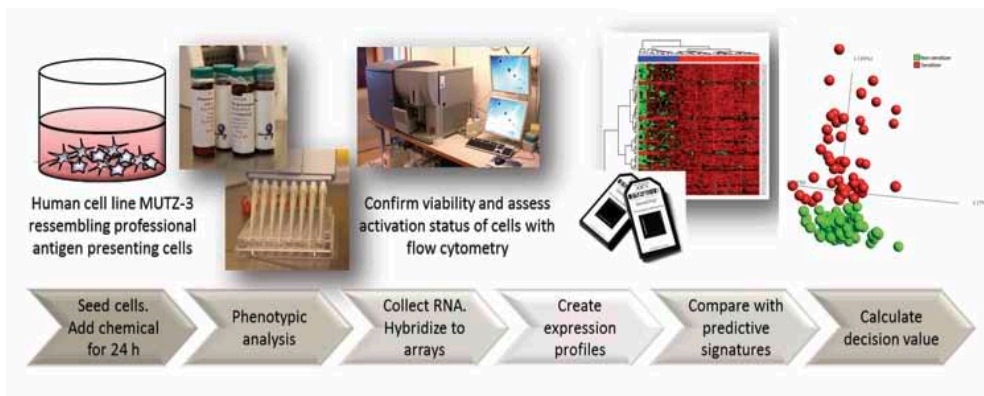
Figure 5. Genomic Allergen Rapid Detection (GARD) test.

3.2. DC Maturation

Tests assessing DC activation (e.g., MUTZ-3, THP-1, U937 cell lines) induction of CD86 (CD54) or IL-8 (protein or mRNA) are currently under pre-validation independently of Sens-it-iv. Sens-it-iv developed a maturation test based upon a CD antibody chip-array for defining CD activation/maturation signatures on DCs. This approach has a medium- to high-throughput, and provides a more descriptive assessment of the compound under investigation. The evaluation studies were concluded, but the analysis of the results remains to be finalised.

3.3. A Novel DC-based Migration Assay

The DC migration assay uses the
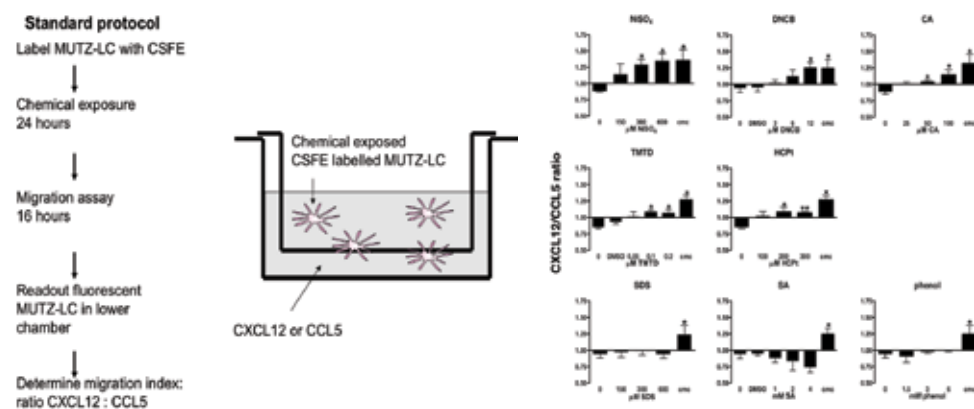
Figure 6. Schematic overview of the trans-well migration assay with representative results.

References: Ouwehand K et al. 2008. Eur J Immunol. 2008; 38, 3050-9; Ouwehand K et al. Eur J Immunol. 2010; 40: 2026-34; Ouwehand K et al. Toxicol In Vitro 2010; 24: 578-85.

Naive T cells
+
iDC + chemical component

Read-out

day -2   **0**   **10/11**

fastDC generation   **+ IL-2 + IL-7 + IL-15**
enhancing in vitro priming and detection of chemical-specific T cells

Flow cytometric detection of IFN-γ and TNF-α by intracellular staining
for reliable identification of *in vitro* primed chemical-specific T cells
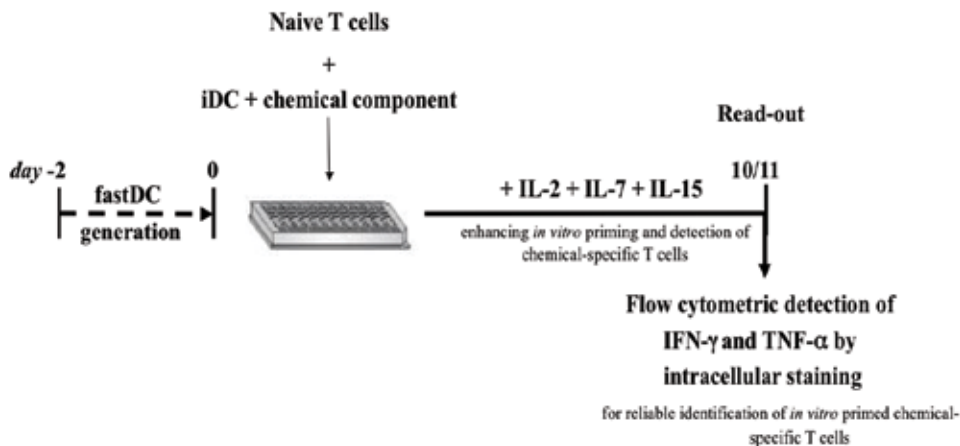
Figure 7. *In vitro* T cell priming assay.

chemokine-specific migration of fluorescently labelled MUTZ-3 Langerhans cells (LCs) in a two chamber system. This functional assay is based upon the migratory behaviour of chemical-exposed MUTZ-LC towards either CXCL12 (sensitiser) or CCL5 (irritant). Currently, no misclassification has been observed with this test.

The DC-migration test is presently further assessed for reproducibility, transferability and reliability by industrial partners (consumer products). The intended application is hazard identification and establishment of a testing strategy for assessing consumer safety.

4. Primary T Cell Stimulation

The development of effector cell-based *in vitro* testing methods that incorporate T_{eff}, NK and T_{reg} cells had to overcome the limitation of low frequencies of allergen-specific precursors in the naïve T cell pool

and to provide allergens in optimal way for presentation by professional antigen presenting cells to the T cells.

Based on the development of efficient methods for the enrichment and amplification of such effector cells and the intense collaboration within Sens-it-iv, the original testing strategies were refined in order to provide effector cell-based *in vitro* assays for allergen testing. Thus, allergen-specific T cell stimulation assays were significantly advanced by efficient techniques to remove regulatory T cells and to simplify the production of autologous DC.

Geiger R, et al. J. Exp. Med. 2009; 206, 1525-34
Carbone T, et al. Immunol. 2010; 184, 1102-10
Dietz L, et al. Toxicol. Sci. 2010; 117, 336-47
Martin SF. Allergologie 2010; 33, 66-70
Martin SF, et al. ALTEX 2010; 27, 309-11
Pennino D, et al. J. Immunol. 2010; 184, 4880-8
Schmidt M, et al. Nat. Immunol. 2010; 11, 814-9
Weber FC, et al. 2010. J. Exp. Med. 207: 2609-19

The T cell-based assays have attracted the attention of the cosmetic industry, and are taken up by the COLIPA Task Force for Skin Sensitisation.

5. Neutrophil Assay for the Prediction of Skin Sensitisation

Levels of IL-8 were systematically found to be higher in the DC line THP-1, when compared to neutrophils, incubated with common sensitising haptens. However, pro-stimulated significantly higher levels of IL-8 secretion from neutrophils. The neutrophil-based assay was able to correctly classify 5 out of 6 pro-haptens as sensitising chemicals.

6. Novel Proteomic Classifiers for Chemical Sensitisation

Large-scale proteomics data exploration resulted in the identification of 120 potential protein sensitisation-specific markers for MUTZ-3 DC. Secreted markers were selected for further verification by antibody-based assay techniques in supernatants of MUTZ-3 cells. New protein biomarkers were found that could be the basis of novel multimarker-based *in vitro* skin sensitisation tests.

Scientific Side-Effects

It was demonstrated and confirmed that contact allergens trigger the same innate immune and stress responses that are activated by microbes, but the mechanisms are different. The term 'xeno-inflammation' was proposed to describe chemical-induced inflammation. The studies have allowed Sens-it-iv to develop a new concept for the innate immune response to contact allergens by drawing analogies to infections. This concept has important implications for our understanding of the pathogenesis as well as for assay development and potential treatment strategies for allergic contact dermatitis (ACD).

From the test development point of view, these basic research studies have influenced the design of *in vitro* T cell-priming assays. They also have pointed out new strategies for assay development based on newly identified innate immune and stress response pathways triggered by contact allergens. An interesting aspect is the close collaboration of the innate immune and stress response pathways. Interference with one of them is sufficient to experimentally prevent ACD. This opens the possibility for preventive strategies in humans and possibly also for treatment of established ACD.

Dissemination & Technology Transfer

Regular activities such as peer reviewed publications (96 published or in press; 5 more submitted), presentations and

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Martin SF, et al. J Exp Med. 2008; 205, 2151-62
Freudenberg MA, et al. G Ital Dermatol. Venerol. 2009; 144, 173-85
Geiger R, et al. J. Exp. Med. 2009; 206, 1525-34
Dietz L, et al. Toxicol Sci. 2010; 117, 336-47
Lass C, et al. Exp Dermatol. 2010; 19, 1007-13
Martin SF. Allergologie 2010; 33, 66-70
Martin SF, Esser PR. ALTEX 2010; 27, 293-5
Martin SF, et al. Cell Mol Life Sci. 2010; 67, 4171-84
Martin SF, et al. ALTEX 2010; 27, 309-11
Schmidt M, et al. Nat Immunol. 2010; 11, 814-9
Weber FC, et al. J Exp Med. 2010; 207, 2609-19

sessions at congress and meetings (7th World Congress, SOT, EuroTox, ITCASS, COLIPA, IVTIP, ecopa), website and newsletters in this area were continued.

In 2011, a congress fully dedicated to sensitisation testing and targeting dissemination and reality check of Sens-it-iv knowledge and expertise is planned. During this congress the Sens-it-iv toolbox as well as tools developed outside the consortium will be discussed. The following charge question will be posed: 'Is our knowledge about skin and respiratory sensitisation sufficient and are the tools available adequate to establish prototype integrated testing strategies?'

Technology transfer focussed on industry, academia and regulatory authorities interested in *in vitro* assessment of the sensitising potency of compounds. To date, technology transfer activities are in process with the Institute for *In Vitro* Sciences (US), Occupational Safety Round Table group (US), BASF, IVTIP, COLIPA, the Danish *In Vitro* Toxicology Network, the Danish Society of Toxicology, Pharmacology and Medical Chemistry. With respect to regulatory authorities, Sens-it-iv has established contact with the Danish Environmental Protection Agency (DEPA), RIVM, EFSA and EMEA.

The concrete outcome of the dissemination activities collaborations with industry (preliminary budget of €120,000) implementing selected tests for product development (cosmetic industry) and REACH (chemical industry). Furthermore a budget of €250,000 was secured for

specific pre-validation studies.

Training & Education

Internal practical training on test performance was continued on *ad hoc* basis, often in the context of assessment of test transferability and reliability. *Sens-it-iv* developed an e-learning tool that has been made available online at *sens-it-iv. eu*.

Next Steps

The aggressive dissemination activities of the last 6 months of the project have resulted in a number of focused projects, which should lead to established implementation of the Sens-it-iv tests, definition of the strengths and limitations of these tests, and acceptance by industry and regulatory authorities. Specific projects include:

1. The two-tiered approach is currently under pre-validation in compliance with the ECVAM guidelines.
2. The two-tiered approach was implemented by the chemical industry for testing chemicals for REACH in a weight-of-evidence approach. Sens-it-iv partners are running these tests.
3. Sens-it-iv was contacted by COLIPA to discuss transfer of the two-tiered approach, which is currently under negotiation.
4. The alveolar and bronchial cell-based tests are presently being further assessed for reproducibility, transferability and reliability by industrial partners (consumer

products and pharmaceutical industries). The intended application is occupational safety assessment.

5. The PCLS approach was taken up for pre-validation by the German government.

6. An additional budget has been secured for the 'respiratory' marker profile to expand the number of compounds (including proteins) and to establish the physiological relevance of the marker profile using the PCLS technology and the MucilAir model.

7. A budget has been assured for the GARD test to be implemented on proteinacous respiratory sensitisers, while negotiations aiming at implementation of the test by certain companies in the cosmetic industry are ongoing.

8. A budget has been assured for DC maturation for evaluation on more compounds and for implementation on proteinaceous respiratory sensitisers. Negotiations aiming at implementation of the test by certain companies in the cosmetic industry are ongoing.

9. A budget was secured for the DC migration test for the assessment of the reproducibility, transferability and reliability by industrial partners (consumer products).

10. The intended application is hazard identification and establishment of a testing strategy for assessing consumer safety.

11. The T cell-based assays have attracted the attention of the cosmetic industry, and are taken up by the COLIPA Task Force for Skin Sensitisation.

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START-UP

Scientific and Technological Issues in Alternatives Research in Drug Development and Union Politics

Grant Agreement Number:	201187 (FP7-HEALTH-2007-1.3-2)
Project Type:	Coordination and Support Action
EC Contribution:	€ 317,964
Starting Date:	1 March 2008
Duration:	24 Months

Website: ecopa.eu

Background & Objectives

The START-UP project must be seen in the context of the actual situation in the EU (and other parts of the world) with respect to the use of '3R' alternative methods to refine, reduce and replace experimental animals in the development process of several product types and their use as a 'safety and efficacy guarantee' for human health. In this project, focus therefore does not lie on alternative methods as such, but on their potential application in the pharmaceutical industry in order to improve the final outcome.

Drug development is an expensive and time-consuming process. Therefore, identifying toxic or non-effective candidates at an early stage and avoiding efforts in optimising under-performing candidates are key for the competitiveness of the European pharmaceutical industry. Bringing in new methodologies and 3R alternatives could provide an important benefit, not only to save animal lives, but also in terms of a more successful drug development outcome. Until today, only a limited list of 3R alternative methods have been officially validated and can be used for regulatory purposes, for example for quantitative risk assessment of cosmetics and their ingredients.

For chemicals in general (i.e., REACH), official validation is not such an issue and alternative methods that are considered to be suitable can be brought into the toxicity dossiers for further evaluation. For the pharmaceutical industry, official validation also seems to be of less importance in the sense that new methodologies or new alternative methods can be applied in basic research, in mechanistic studies and in all stages of preclinical research before results are confirmed by a limited number of animal studies,

bridging the gap to human volunteer studies.

Basically, no major restrictions exist in this field as long as the 3R methodologies used are scientifically sound and relevant, have elucidating and discriminative power, and seem of interest at a particular stage of drug development. Therefore, a project such as START-UP was necessary, namely a co-ordinated initiative covering as much as possible all parties involved. Important stakeholders in this project are of course the pharmaceutical industry, scientists and researchers, animal welfare representatives, and regulatory bodies involved in pharmaceutical agencies in order to achieve a major collaborative activity. It is important to build up a realistic overview of the current use of experimental animals in the drug development process and to assess the possibility to implement new alternative strategies and tiered approaches in the different stages of the drug development process. The task of identifying existing gaps, scientific and technological bottlenecks, ethical concerns and issues related to Union politics has been carried out with success. Besides new chemical entities, biopharmaceuticals or new biological entities were also included in the study. Biological drugs such as monoclonal antibodies, peptides and proteins are becoming increasingly important. The same is true for nanotechnology and nanobiotechnological molecules. This creates new challenges as safety clearance of these new types of substances is more complex than is the case for traditional low-molecular-weight chemical

substances. Safety testing in animals is in certain cases not even relevant since only a limited number of relevant species exist that can give answers on immunological events and antibody formation. On the contrary, up-to-date 'fingerprint' techniques may offer possibilities to better target the problems and mechanisms involved so that only relevant molecules on a limited number of animals of the relevant species need some testing in order to guarantee safety and efficacy.

The direct objectives of this project consisted of:

- Gathering all relevant information, mentioned above, by organising two expert meetings with pharma- and biotech-experts and 3R specialists (note that an additional third expert meeting was organised
- Prioritisation of this information within the three domains of refinement, reduction and replacement
- Organisation of three high-level workshops, one on each of the 3Rs
- Developing a consensus report between all parties involved on the outcome of the expert meetings and workshops
- Proposing roadmaps for the European Commission.

All these objectives have been met within the given limited timeframe of 2 years.

Deliverables & Milestones Achieved During 2010

As the project was officially ran from 1 March 2008 until 28 February 2010, the

practical work of organising 3 'closed' experts meetings (in Madrid, Basle and Alicante) was carried out already in 2008. The follow-up of 3 'open' workshops ended also already in 2009—namely, a Refinement Workshop which took place in Rome (26-27 February 2009), a Reduction Workshop in Innsbruck, Austria (3-4 July 2009), and a Replacement Workshop in Budapest (2-3 October 2009).

Thus, all practical work foreseen in the project was carried out before the end of the project. All reports of the individual workshops were made by the Co-ordinator and corrected by all stakeholders involved. At the official end of the project, a final report, including an executive summary, recommendations and roadmaps were sent to the European Commission for final improvement. Due to the introduction of new computer programmes and new website links at the EU level, there was some confusion and it took several months before all documents were officially cleared.

The budget, necessary for a final booklet dedicated to the project, was not present anymore after the official ending of the project. However, a special effort was made by the responsible team of the Co-ordinator to still make an attractive booklet with the official report present in attachment on a CD. This was finalised by the beginning of January 2011 and is now available for distribution. The booklet is being distributed now to all participants of the project (223 persons in total), other scientists and organisations. It contains a number of topics that should be taken up as much as possible in future projects within the EU Framework Programmes. They can be summarised as follows:

Collation of 3R Topics in Pharmaceutical Research

- Animal experiments are still needed and realistic progress is expected by intelligent combination of refinement, reduction and replacement methodologies/strategies. This is, in particular, relevant in animal disease models. *In vivo* and *in vitro* research and testing should go together and not be seen as two opposites.
- It was emphasised that an alternative method does not necessarily need to be formally validated; the fact that a test is useful in the pharmaceutical industry is of more importance.
- Data obtained from *in vitro* tests, carried out before *in vivo* experiments start, can efficiently filter compounds of interest. These pre-tests should be of a higher degree of sophistication and complexity than is the case now (e.g., use of 3D-cultures, co-cultures, stem-cell derived models, organ-specific and differentiated cell cultures); more human cells use and more attention for the parameters measured (e.g., it is unlikely that only one biomarker will cover the complexity of the living organism), therefore a set of specific biomarkers of clinical relevance increases the translational nature of the *in vitro* model used; these should be developed at least for key organs and new and potent tools should be involved (e.g.,

combinations of transcriptomics, metabonomics, biostatistics).

- When animals are involved, they should be of a relevant species for the question posed; otherwise, experimentation should be deleted. The same is true for exposure to unrealistically high dosages and exposure scenarios.
- Fields for further development are teratogenicity and embryotoxicity, as these tests are necessary for every newly developed drug coming on the market and they consume a high number of animals. For exploration of new opportunities for pharmacodynamics, and for a better integration into single test programmes for pharmacokinetics, carcinogenesis, safety pharmacology and toxicology seem to be important.
- In test development more focus should be on 'quantitative risk assessment' rather than on 'hazard assessment'.

Cell System Improvements

- These were high on the agenda. Stabilisation (e.g., by epigenetic modifications, miRNA interaction) of existing cell systems and use of these for long-term testing has potential for toxicity and efficacy testing. In addition, the fact that the heterogeneity of human population is not taken up by current *in vitro* tests deserves efforts to develop models capable of mimicking human variability.

Data Sharing & Reporting of 'Negative' Results

- These aspects are important in gaining more basic information and reducing replication of experiments. They are of special importance in a number of specific diseases.
- Essential for sharing data are data quality control, protocol standardisation and in particular protection of intellectual property. It was proposed to overcome this hurdle by establishing a 'neutral' pan-European party entity.

Animal Husbandry Best Practices

- Emphasis was given to positive aspects such as better training of personnel and in particular of Competent Authorities; positive welfare of experimental animals, e.g., via group housing, creation of possibilities for natural behaviour, environmental enrichment, consideration of positive reinforcement training in the case higher animals are involved
- Proposals for central breeding of controlled and certified quality were particularly brought forward for primates and transgenic animals.
- Emphasis was also given to the importance of the microbiological quality of the animals, leading to better experiments and indirectly leading to less animal use.

Furthering of Model Development, Especially of Non-Invasive In Vivo Methodologies

- This point came up in all meetings and workshops and supports the further transfer of non-invasive diagnostic methodologies (e.g., magnetic resonance imaging, micro-CT) from human medicine to laboratory animals allowing not only diagnosis, but also long-term monitoring of treatment. In particular, the combination of different non-invasive imaging techniques was seen as a possibility for refinement and reduction and at the same time for gaining better knowledge.
- In particular, in animal disease models, this methodology is seen as a key improvement.

Bottlenecks in Biologics Development

- Use of humanised models, knock-out animals and transgenic animals could help to make more appropriate use of animals as high target specificity is involved. Also transgenic cells/enzymes/*in vitro* models have relevance.
- More parameters should be combined in one animal study (e.g., safety pharmacology, pharmacokinetics, local toxicity, immunogenicity).
- Standardisation of animal strains, microbiological high quality of animals, use of well-defined environmental conditions and techniques are crucial reduction parameters in this field.

Vaccine Quality Control

- Regulatory authorities request that all vaccines must be tested, and this consumes a large number of animals. Moving from this traditional quality control concept towards the monitoring of all crucial steps during production could save these animals. This so-called 'consistency approach' was largely supported.
- In vaccine quality control, refinement strategies should be developed and implemented.
- Implementation of existing 3R methods should be encouraged by improving the global harmonisation of the regulatory procedures. Also providing incentives for development and production is considered to be important.
- More attention should go to the neglected area of veterinary vaccines.

Specific Animal Disease Models with High Welfare Burden

- As animal pain models are not very predictive, well-controlled studies in humans using micro-dosing were proposed in order to be able to score pain in a realistic way.
- Animal models of cancer are also a special target for further improvement, since by the development of biologics for this topic, the area is better covered. In oncology, genetically engineered models and primary tumour models were said to be productive. A refinement alternative could be the study of surrogate tissues from

normal animals who usually exhibit the fully functioning pathways that are targeted. Also, the translational aspect, namely the importance of measuring *in vitro* specific biomarkers that can also be detected in the clinical situation, came up.

Analysis of Union Politics & Country/Member States Politics

- Over-expectations with respect to alternative methods should be avoided
- Ethical issues and political restrictions were discussed with respect to human stem cell use. Heterogeneous opinions within the different Member States should be better harmonised
- Member States should establish National Animal Welfare and Ethics Committees with well-trained personnel to give advice to the competent authorities and permanent ethical review bodies of establishments. Networking of these committees should play a role in the exchange and communication of best practices
- Importance was given to a trans-sector, cross sector-cutting information stream by regulators and industrial partners.

Refined Analysis of General EU Research Strategies

- The general research strategies applied today at the EU level are a burden to potential applicants and the administration of EU Framework Programmes are seen as a hindrance to appropriate research in alternative

methods. Less bureaucracy, better integration of research teams, eventual leadership by the pharmaceutical industry, limitation of number of projects per team and need for new names of young scientists and a fresh outlook were all mentioned as possible improvements.

Global Harmonisation

- The importance of global harmonisation as the basis for further implementation of alternative methods came up in all meetings and workshops.
- A unified animal legislation and, in this context, specific actions addressed towards the political world were seen as important.
- Communication on new models across sectors, involving regulatory agencies and Competent Authorities should be enhanced.
- Dissemination and promotion of refinement/reduction techniques in drug development was seen as an important step forward.
- Global harmonisation is highly important and should be pursued even if it is difficult and slow.

Worldwide harmonisation should be brought in the execution of pharmaceutical registration and general concepts, also existing animal welfare in the different Member States should be better harmonised, and the revised Directive on the protection of experimental animals could help in this process.

Conclusions

In summary, the FP7 project START-UP has delivered a whole landscape of ideas and potential avenues for further research and development projects within the future EU Framework Programmes in regard to 3Rs bottlenecks and EU industry competitiveness. These should be considered when drawing up new project calls in this area in the future. It has been demonstrated that only detailed discussions with experienced experts can lay groundwork for adequate analysis.

In the detailed report, a list of specific recommendations is given for each expert meeting and the 3R workshops, making a total of 36 recommendations for further follow-up by the parties concerned and in particular by the Commission. Also a clustering picture of the issues identified in the *ecopa* START-UP project is foreseen (Figure 1). Also relevant workshop proposals ahead are brought together in a comprehensive scheme (Figure 2).

Recommendations

1. Reduction and refinement are particularly possible in the field of animal disease models. It is recommended to maximise the number of non-invasive and early or surrogate endpoints within one model. Progress in non-invasive test
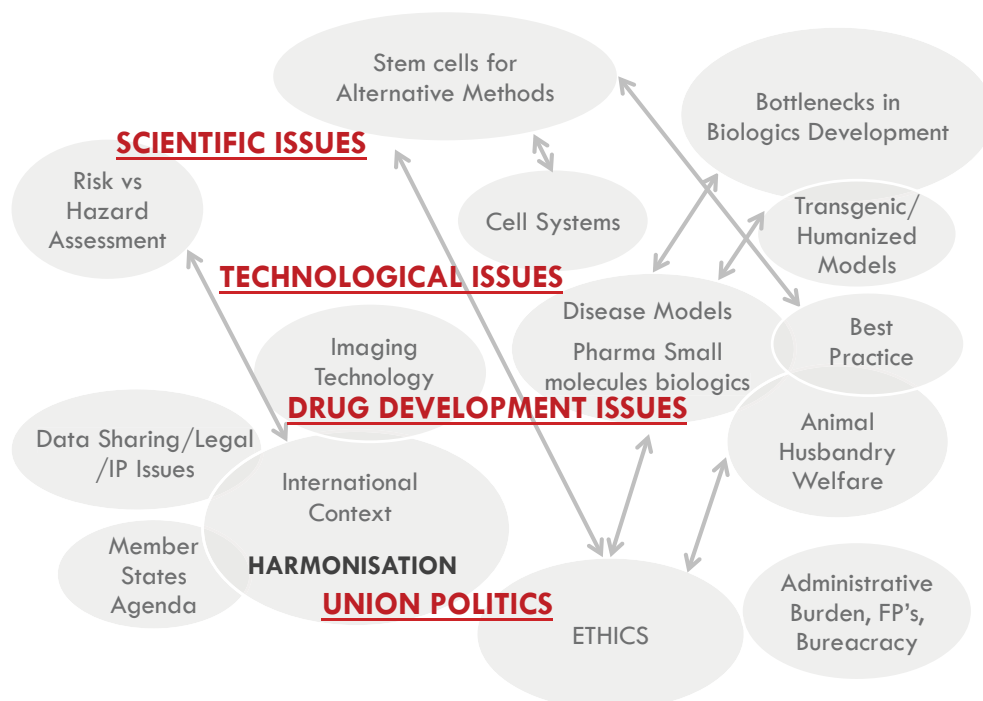
Figure 1. Clustering of issues identified in the START-UP project.

Workshop proposals
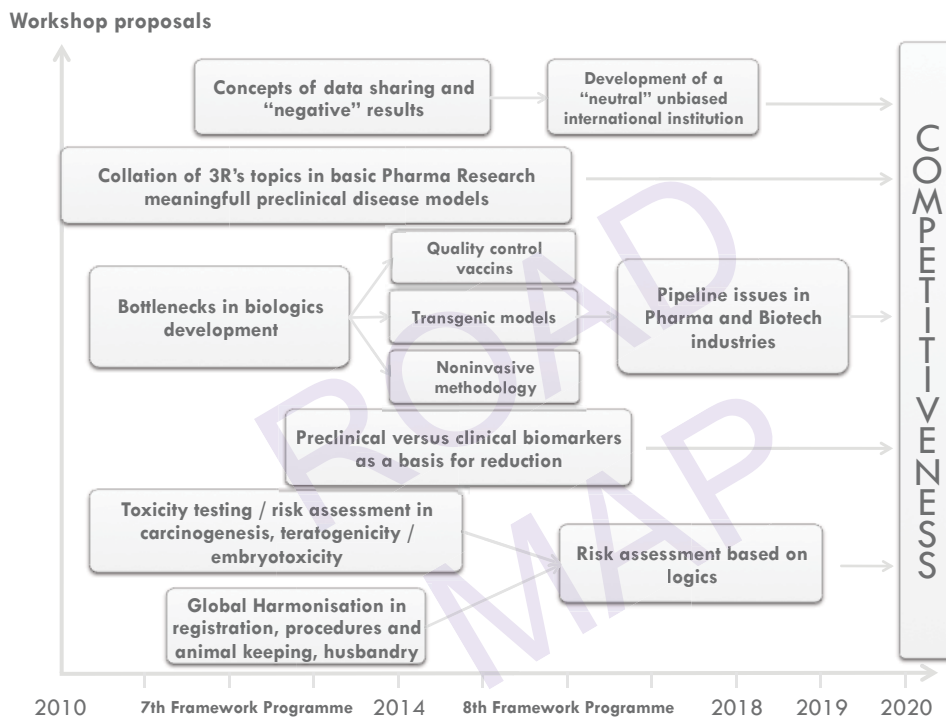
Figure 2. Workshop proposals.

development is seen in the further development of non-invasive imaging/ diagnostic techniques transferred from human medicine to laboratory animals, and their intelligent combination.

2. Efforts should be focused on the development of batteries of sensitive and specific safety biomarkers with clinical relevance to be measured during the preclinical in vitro testing phase.

3. The difference in bottlenecks during the development of biopharmaceuticals versus small molecules pharmaceuticals should be better recognised and dealt with. In particular, the relevance of

animal models came up in the case of biopharmaceuticals. The use of non-human primates (in a number of indicated cases), humanised models and transgenic animals seem relevant.

4. A lot of animals could be spared without loss of quality in the quality control of vaccines in Europe. It is therefore highly recommended to study the possibility for drastic change:

- by a better control of the implementation of already existing refinement and reduction alternatives by all producers and regulatory bodies
- by providing the necessary

incentives to apply these alternatives
- by stimulating the development of new alternatives in this field
- by applying the so-called consistency approach confirming production consistency
- by paying special attention to veterinary products.

5. It is recommended to develop the possibilities of 'data sharing' by creating the necessary working tool, namely the establishment of a 'neutral' non-biased body that could guarantee confidentiality and as such could take away the fear of losing competitiveness. In this way also, quality control of data and standards of protocols could be assured.
   Furthermore, it was felt that the follow-up of 'negative' results of high standard could contribute to the reduction process.

6. Animal reduction in drug development is possible by reducing the number of potential interesting molecules that undergo in vivo testing by better pre-screening for unwanted effects and deceiving efficacy.
   Therefore, more sophisticated in vitro models based on human cells and tissues should be developed and applied in pre-screening, e.g., 3D-models, co-cultures, epigenetically stabilised cell lines, stem-cell derived specific cell types, etc.

7. Promotion of positive animal welfare, in addition to minimalisation of suffering, is seen as a refinement priority and should include active improvement of the degree of animal welfare both during and outside of experimental procedures, backed up by ethological studies animals in laboratories.

8. Global harmonisation is seen as one of the highest priorities for further success in the implementation of the 3Rs. It is thought that all different players internationally involved in drug development, human health, alternative methods development, and animal welfare should be brought together to agree on the different procedures to be followed in registration toxicity and efficacy testing, and risk assessment, in the development of biologics and quality control of vaccines, and in the different stages of animal use during drug development, in particular in the case of animal disease models.

Publications

The publication of a booklet incorporating a CD with the full report is now done. It includes the main project results and will be distributed via the START-UP network and during relevant meetings and workshops. 500 copies are foreseen.

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VITROCELLOMICS

Reducing Animal Experimentation in Preclinical Predictive Drug Testing by Human Hepatic *In Vitro* Models Derived from Embryonic Stem Cells

Contract number: LSHB·CT·2006·018940
Project type: Specific Targeted Research Project (FP6)
EC contribution: € 2,942,000
Starting date: 1 January 2006
Duration: 42 months
Website: er-projects.gf.liu.se/~vitrocellomics

Objectives

The objective of the project was to establish stable cell lines that reliably reflect human hepatic properties by the development of *in vitro* models derived from human embryonic stem cells (hESC). The aim has been to deliver such reliable *in vitro* models that they could be used by the pharmaceutical industry in order to replace experimental animals in investigations on liver toxicity, drug metabolism, uptake and efflux properties of compounds in the drug discovery and development processes. In the pharmaceutical industry, reliable *in vitro* cell models have potential to replace current techniques and animal experimentation in the selection and optimisation of lead compounds and in documentation of a selected drug candidate before it enters clinical phases.

The overall objectives of the VITROCELLOMICS project were:

- Replacing animals in preclinical pharmaceutical development by human hepatic culture systems
- Supporting the predictability of the drug discovery and development process by allowing more reliable and relevant testing in the preclinical phase and hinder weak lead candidates to enter clinical phases with innovative human hepatic cell systems
- Delivering an *in vitro* testing system with adjacent methodology pertinent for validation in GLP/SOPs environment for absorption, metabolism, and toxicity
- The ultimate aim was to markedly reduce the use of animals in drug testing,

refine the model system under consideration and to replace the animals currently used.

The clinical expertise in the project was manifested by the involvement of two renowned European university hospitals, one SME founded by another well-known European university hospital and the partnership with one of the leading European pharmaceutical companies. Three partners represent a solid reputed bioengineering background. Other important partners were one SME focused on development of standardised assay conditions for drug testing, and the European Centre for Validation of Alternative Methods (ECVAM).

Main Results

The results of the VITROCELLOMICS project are reviewed in volume 35 of *Alternatives to Laboratory Aniamls* (Mandenius et al., 2011). Full reporting of project results is available in the references 2-20 under Publications.

A significant achievement of VITROCELLOMICS has been establishment of protocols for direct differentiation of hESC lines towards mature hepatic phenotype, which have been continuously modified, refined and tested. A milestone
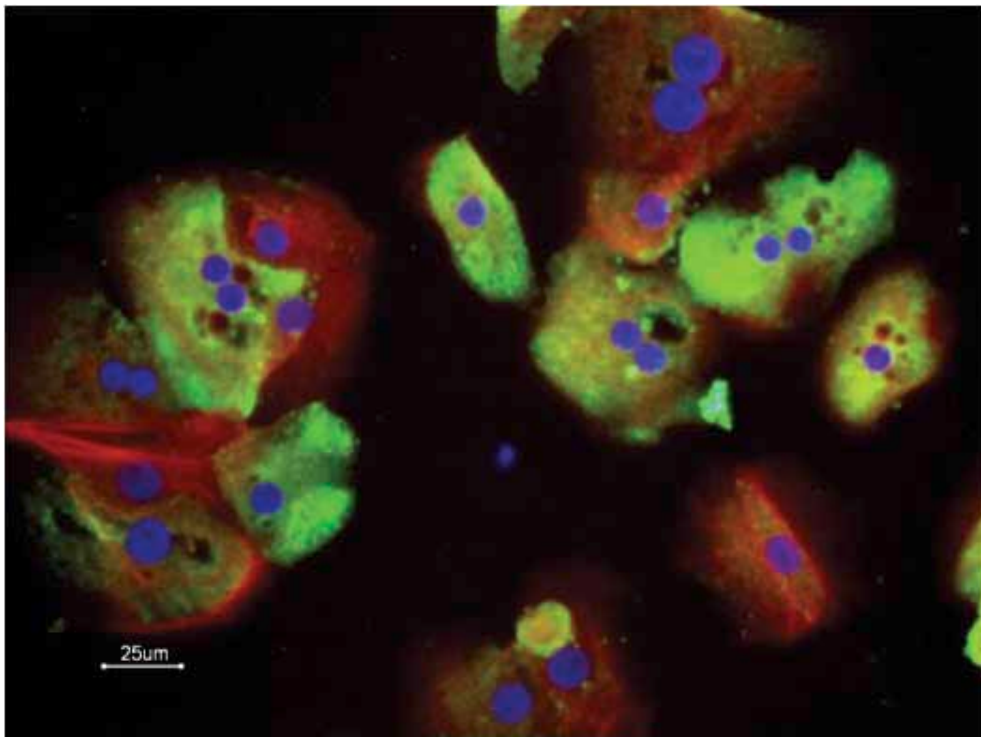

Figure 1. Immunocytochemical staining of hESC-derived hepatocyte-like cells. Positive staining for metabolising enzyme CYP3A4 and liver marker CK18 are shown in green and red colour respectively. DAPI staining for cell nuclei is shown in blue.

resulting of the project was the ability to control the hepatic differentiation via the germ layer definitive endoderm further to functional hepatocytes. Moreover, these derived cells display enzyme activities closer to mature liver than attained with previously derived cells, better clearance properties of drugs and more distinct expression of biomarkers characteristic for mature liver functionality (Figure 1).

Generation of toxicity and drug metabolism data have been completed with the selected test compounds using assays evaluated and selected earlier in the project. Assays used have been optimised for hepatocyte reference cells in the partner laboratories. It was concluded that established toxicity assays are rather poor predictors of clinical toxicity and that hESC-derived cells may have much higher potential for desired level of predictability. Furthermore, it was demonstrated that the toxicity test method with optical sensing of oxygen consumption (respiration assay) is well suited for incorporation in the project's toxicity test platform.

The partners working on test method development used the improved hESC-derived hepatocytes in order to optimise the methods with these cells and to compare toxicity and metabolism with the data from the reference cell types.

Respiration

Respiration was measured in both 96-well Oxoplates and 24-well OxoDishes (Figure 2), which are based on the measurement of luminescence intensity and decay

time respectively. Both methods are non-invasive and allowed the monitoring of toxicity online, thereby allowing the assessment of the toxicodynamics of the toxic effect of the tested compound.

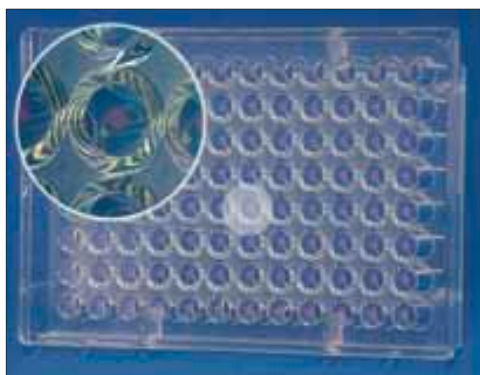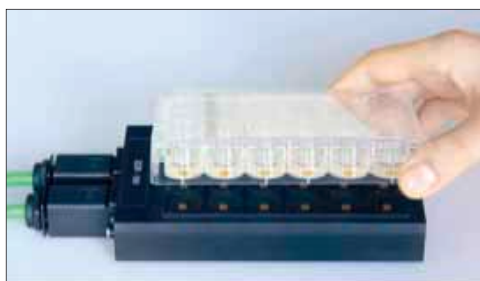Respiration in reference cell line (Hep G2) was measured continuously for a

Figure 2. SensorDish reader with OxoDish (A). Setup of multiple OxoDishes connected in series in a humidified cell culture incubator (B). A 96-well Oxoplate (C).

desired period of time and EC_{50} values were calculated at any chosen time point. There was a good correlation with other commonly used endpoint assays. All the selected drugs were screened on the reference cell line. It was concluded that Hep G2 cells allow 100% prediction of parent compound toxicity when used in a multi-assay platform with at least one kinetic assay.

Respiration measurements using hESC-derived hepatocytes, cryopreserved primary hepatocytes and Hep G2 in 24 well respiration assays were also carried out. Diclofenac and amidoarone were tested in a range of concentrations for the determination of the EC_{50} values.

Fluxomics

Metabolite balancing and ^{13}C labelling studies were carried out. Using metabolite balancing a flux map was established for Hep G2 cells. A simplified stoichiometric network model based on the experimental data obtained by exometabolome analysis (measurement of uptake and secretion of metabolites) and determination of the biomass composition of Hep G2 cells was established. Further information from labeling experiments giving the pentose phosphate pathway activity was included. Using [1, 2-^{13}C]-glucose, the labeling in lactate was quantified and the mass isotopomer ratios were calculated to estimate the pentose phosphate pathway (PPP). The PPP activity was 4.7 % without exposure to a test compound. The activity was 4.6% upon exposure to 50µM tacrine.

It is noteworthy that the changes observed in the fluxes were at drug exposure concentrations at which there was no change in the viability of the cells as well as no change in the glucose uptake. Metabolic flux analysis is therefore, a very sensitive method to identify toxicity at a very early stage before any effect could be measured by classical endpoint methods.

Cultivation conditions in small-scale bioreactors have been further evaluated and adapted using primary hepatocytes. The optimisation made resulted in markedly higher survival rates when hepatocytes were cultured as 3D structures. Also, improved maintenance of hepatocyte functionality in three-week cultures was obtained, showing more that 10-fold higher in UGT activity and 3-fold higher ECOD activity (Miranda et al., 2009). Furthermore, optimisation of 3D bioreactor cultivation for the purpose of large-scale expansion of cells is in progress, at present, using mouse ESC. Partners working on 3D bioreactor cultivation tasks are receiving hESC-derived hepatocytes for further optimisation of cultivation procedures.

Of special interest are the studies carried out in the four-compartment artificial liver bioreactor. The introduction of a downscaled bioreactor prototype in combination with partial automation of the perfusion periphery enabled studies on hepatocyte metabolism with reduced cell numbers in a controlled environment. Optimisation experiments of the system have been conducted in the project with various hepatic cells and cell lines under
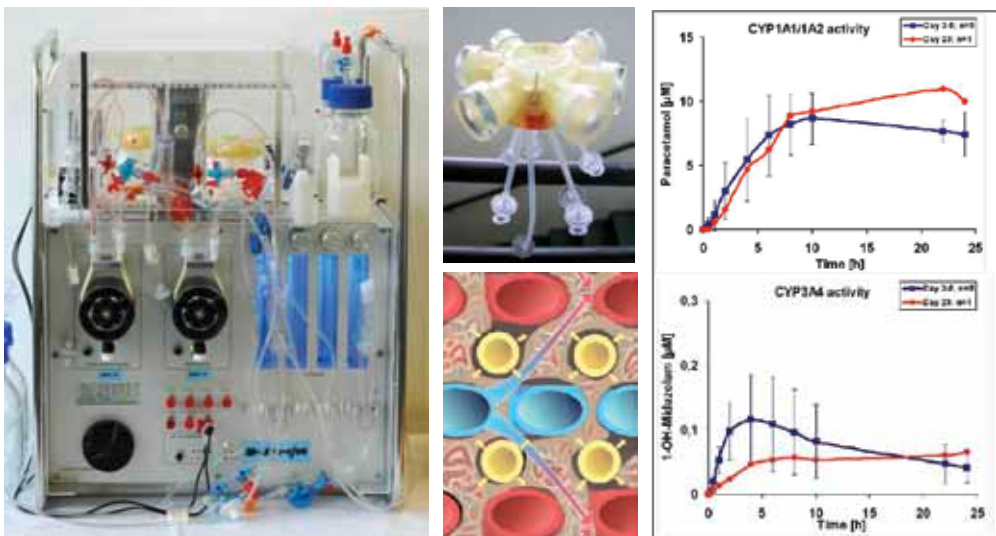
Figure 3. Four-compartment artificial liver bioreactor with three independent capillary bundles for culture medium perfusion (red and blue) and oxygenation (yellow) of the cells located in the extra-capillary space. Long-term stability of cytochrome P450 enzyme activities was demonstrated in human hepatocyte bioreactors.

varying conditions. Basic culture media optimisation has been an integral part of the studies (Dong et al., 2008). Using suitable reference substrates, stable maintenance of drug metabolising enzymes in liver cell bioreactors was demonstrated. The system was also successfully used for expansion of mouse embryonic stem cells (mESC) used as a model cell line (Gerlach et al., 2010). Studies on spontaneous differentiation of mESC in the bioreactor showed the ability of the bioreactor system to support cell-specific maturation of ESC *in vitro* (Gerlach et al., 2009). Similarly, long-term cultivation of hESC in bioreactors led to the formation of tissue structures containing cell types of all three germ layers, similar to teratoma formation *in vivo*. Studies on directed hepatic differentiation of hESC in the four-compartment bioreactor are in progress.

A method on metabolic liver enzyme (CYP) induction was pre-validated using HepaRG reference cells provided by BioPredic. The structure of the pre-validation study was based of the modular approach proposed by ECVAM (Hartung et al., 2004). The pre-validation study was performed by Pharmacelsus (lead laboratory), IBET and ECVAM (naïve laboratories).

The pre-validation contained four phases:

1. In the first phase, within-batch reproducibility was tested
2. In the second phase, between-batch and within-laboratory reproducibility was evaluated
3. In the third phase, between-laboratory reproducibility will be tested

4. Based on the results from the three phases, the validation management group will decide about the study design about the fourth phase of the study (predictive capacity)

Publications 2010-11

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embryonic stem cells differentiating to definitive and primitive endoderm and further toward the hepatic lineage. Stem Cell Develop. 2010; 19, 961-78.

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Patent Applications Filed

1. Integration of a Cytochrome P450 induction assay on primary human hepatocytes to the Pharmacelsus portfolio.
2. Integration of the (pre-validated) method for Cytochrome P450 induction on the HepaRG cell line to the Pharmacelsus portfolio.
3. A novel population of hepatocytes derived via definitive endoderm (DE-hep) from human blastocysts derived stem cells. Patent filed July 2007 – US60/935,003 by Cellartis AB.

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3

AXLR8-2 WORKSHOP REPORT

3.1 Overview

The second annual AXLR8 workshop (AXLR8-2) was held in Berlin, Germany from 22-25 May 2011 with a focus on developing a 'roadmap to innovative toxicity testing'. Among the more than 50 invited participants were representatives of projects funded by the FP6 and FP7 health and environment programmes, the heads of EU Member State centres on alternatives to animal testing, the leaders of international efforts to establish advanced molecular toxicology from the United States and Japan, and members of the AXLR8 Scientific Panel and Consortium.

The workshop began with a public satellite meeting, providing an overview of current EU and global research efforts such as the joint initiative between DG R&I and the European Cosmetics Association (COLIPA) aimed at 'replacement of in vivo repeated dose systemic toxicity testing' with the long-term target of 'safety evaluation ultimately replacing animal testing' (hereafter referred to as 'SEURAT'), and the work by regulatory and research agencies in the United States aimed at 'toxicity testing in the 21st century'. The satellite meeting was also devoted to innovative disease models based on mapping of molecular and cellular 'pathways' of human disease; to advanced methods funded by the German Ministry of Research and Technology such as the 'virtual liver' project and a multi-organ chip project; and to the European Medicines Initiative 'eTOX' project on the use of proprietary pharmaceutical industry data in bioinformatics.

The formal workshop programme included updates from FP6/7 projects, European companies and academic scientists, and international 'thought leaders'. Plenary presentations focused on the toxicity pathway concept in general, with case studies in the areas of reproductive toxicity and sensitisation (allergy). Workshop slides are available online at *axlr8.eu/workshops/axlr8-2-workshop.html*, and presentation manuscripts are included in Section 3.3 below.

Workshop participants were divided into breakout groups for a focused discussion of the scientific state-of-the-art and of knowledge gaps and priorities for future EU research funding. Reports from these groups are provided in Section 3.4. The workshop concluded with an *in camera* (closed) meeting of the AXLR8 Scientific Panel aimed at refining a strategy and roadmap for future EU research in this area, with a clear focus on advancing the 'Europe 2020' goals of addressing major societal challenges through high-impact, results-driven research and robust integration of key technologies in the field of health (and environmental) protection. The results and recommendations of the AXLR8-2 workshop are summarised in Section 3.5.

3.2 Scientific Programme

AXLR8-2 WORKSHOP: ROADMAP TO INNOVATIVE TOXICITY TESTING

SEMINARIS CAMPUS HOTEL BERLIN

SUNDAY, 22 MAY 2011

Satellite Meeting on Innovative Methods

15.00 – 15.10	Welcome & Opening Remarks
	Monika Schäfer-Korting & Horst Spielmann, AXLR8 & Freie
	Universität Berlin, DE
15.10 – 15.40	The EU-COLIPA SEURAT-1 Project: Towards the Replacement of *In*
	Vivo Repeated Dose Systemic Toxicity Testing
	Michael Schwarz, University of Tübingen, DE
15.40 – 16.10	The Human Toxicology Project
	Andrew Rowan, Humane Society International, US
16.10 – 16.40	TOX21 and Disease Pathway Integration
	Raymond Tice, National Institute of Environmental Health Sciences,
	US (presentation given by Robert Kavlock, Environmental Protection
	Agency, US)
16.40 – 17.10	Human Skin Disease Models
	Monika Schäfer-Korting, Freie Universität Berlin, DE
17.10 – 17.30	Coffee Break
17.30 – 18.00	BMBF 'Virtual Liver' Project
	Jan Hengstler, IfADo, DE
18.00 – 18.30	BMBR Go-Bio Project 'From Single Tissue Culture to Multi-Organ
	Chips'
	Uwe Marx, Berlin TissUse & TU Berlin, DE
18.30 – 19.00	The IMI 'eTox' Project: Efforts to Develop *In Silico* Tools to Predict *In*
	Vivo Toxicity
	Thomas Steger-Hartmann, Bayer Schering, DE
19.00	Close of session

MONDAY, 23 MAY 2011

Progress Reports from FP6/7 Projects
Chairs: Manuel Carrondo & Michael Schwarz

08.30 – 08.45	Welcome
	Horst Spielmann, AXLR8 & Freie Universität Berlin, DE

08.45 – 09.15	ACuteTox
	Annette Kopp-Schneider, DKFZ, DE
09.15 – 09.45	NanoTest
	Maria Dusinska, NILU, NO
09.45 – 10.15	OpenTox
	Barry Hardy, Douglas Connect, CH
10.15 – 10.30	Coffee Break
10.30 – 11.00	ESNATS
	Marcel Leist, Universität Konstanz, DE
11.00 – 11.30	Predict-IV
	Christof Burek, Universität Würzburg, DE
11.30 – 12.00	START-UP
	Peter Maier, University of Zürich, CH
12.00 – 13.00	Lunch Break

Case Study 1: Reproductive Toxicology
Chairs: Robert Kavlock & Ellen Fritsche

13.00 – 13.30	Genetic Causes of Congenital Cardiac Defects
	Silke Sperling, Charité & MDC, DE
13.30 – 14.00	Tox21: ToxCast & ToxPi Projects
	Robert Kavlock, Environmental Protection Agency, US
14.00 – 14.30	EU FP6 Project ReProTect
	Michael Schwarz, University of Tübingen, DE
14.30 – 15.00	Protein Biomarkers for Embryotoxicity
	André Schrattenholz, Proteosys, DE
15.00 – 15.15	Coffee Break
15.15 – 15.45	ES Cells and Molecular Endpoints
	Aldert Piersma, RIVM, NL
15.45 – 16.15	The FP7 Project CHEMSCREEN
	Bart van der Burg, BDS, NL
16.15 – 16.45	The US EPA Virtual Embryo Project
	Tom Knudsen, EPA, US
16.45 – 17.00	Coffee Break

Case Study 2: Skin Sensitisation
Chairs: Nathalie Alépée & Robert Landsiedel

17.00 – 17.30	The GARD Test
	Malin Lindstedt, Lund University, SE
17.30 – 18.00	Gene Expression in Dendritic Cells: From Statistical Modelling to Pathway Discovery
	Jef Hooyberghs, VITO, BE
18.00 – 18.30	Modelling the Human Adverse Response
	Gavin Maxwell, Unilver, UK

18.30 – 19.00	The OECD Adverse Outcome Pathway Approach
	Steven Enoch, University of Liverpool, UK
19.00	Close of session

TUESDAY, 24 MAY 2011

Morning Session

Chairs: Jürgen Büsing & Andrew Rowan

08.30 – 09.00	Transcriptional Response to Chemicals
	Morgane Thomas-Chollier, MPI Berlin, DE
09.00 – 09.30	EU-COLIPA Project SEURAT-1: Towards the Replacement of *In Vivo* Repeated Dose Systemic Toxicity Testing
	Michael Schwarz, University of Tübingen, DE
09.30 – 10.00	The Japanese METI-NEDO High Throughput Assay Systems Project
	Noriho Tanaka, Hatano Research Institute, JP
10.00 – 10.15	Coffee Break
10.15 – 10.45	Progress Toward Toxicity Testing in the 21st Century
	Harvey Clewell, Hamner Institutes, US
10.45 – 11.15	Collaboration Between the JRC and Tox21
	Maurice Whelan, JRC-IHCP, IT
11.15 – 11.45	Safety Assessment at Unilever Applying the Tox21 Approach
	Julia Fentem, Unilever, UK
11.45 – 12.15	Co-ordinating Actions and Research Policy
	Jürgen Büsing, European Commission, BE
12.15 – 13.15	Lunch Break

Afternoon Session

Chairs: Julia Fentem & Maurice Whelan

13.15 – 17.30	Breakout Groups
17.30 – 18.30	General Discussion
18.30	Close of session

WEDNESDAY, 25 MAY 2011

Closed Meeting of AXLR8 Consortium & Scientific Panel

Chairs: Julia Fentem & Maurice Whelan

09.00 – 11.00	Review of Recommendations from BOGs 1-3
11.00 – 11.15	Coffee Break
11.15 – 13.00	Draft Workshop Report
13.00	Close of Workshop

3.3 Workshop Presentations

Accelerating the Transition to 21st Century Toxicology

The Work of the Human Toxicology Project Consortium

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Introduction

There is growing frustration with current methods of toxicity testing for human health risk assessments. These approaches date to the 1930's, with only incremental changes over time, and decisions about likely human effects of chemicals are based on high dose, generally long-term studies in rats and mice. These tests are time-consuming and resource intensive (Table 1), and their relatively poor predictive power is indicated by the fact that a safety factor of 100-1,000 is typically applied when estimating safe human exposure levels. These characteristics mean there are significant restrictions on the number of chemicals that can be tested in any one year. Furthermore, because the established toxicity tests are, to a considerable degree, based on a 'cookbook' approach to evaluating specific endpoints, the recorded and submitted results of such tests typically provide little understanding of the mechanistic underpinnings of how chemicals act in the body.

For the past thirty years, there has been an increasing interest in the potential to replace or reduce animal use in toxicity testing. Typically, the approach has involved seeking cell-based assay systems that are able to predict the endpoint identified in the animal, e.g., a cell-based cytotoxicity assay to predict skin irritancy. While this approach—'Alternatives 1.0'—has had some success, it has also proven to be slow and somewhat cumbersome. Typically, the whole process from research and development through validation and regulatory acceptance and implementation takes at least 10 years. The resulting tests are still typically only 'medium-throughput' and they are also still based on comparisons

Table 1. A sampling of current toxicity test requirements.

Endpoint	OECD TG	Started	Species 1	# animals	Cost ($)	Species 2	Duration
Cancer	451, 453	1962	Rat	400-500	700K-3M	Mouse	2 yrs
Irritation-dermal	404	1944	Rabbit	3	1,800	NA	2 wks
Irritation-ocular	405	1944	Rabbit	3	1,800	NA	2 wks
Neurotox: Develop.	426	Early 1960s	Rat	1,400	417,000	NA	4 mos
Repro: 2-gen	416	Early 1960s	Rat	2,600 – 3,200	380,000	NA	8 mos
Dermal sens.: GPMT	408	?	Guinea pig	32	6,000	NA	3 wks
Systemic: Acute Inhal.	403 433 436	1927	Rat	40	16,000	NA	3 wks
Systemic: chronic	452	?	Rat	160	700,000	Dog	1-2 yrs

with animal data as the gold standard. Given the existing problems with animal data, this is not particularly satisfactory. It is also difficult to see how this approach might begin to address chronic toxicity endpoints. According to the Interagency Coordinating Committee for the Validation of Alternative Methods, only 35 alternative tests, of which 23 are *in vitro* systems, have been accepted as scientifically valid for use in the US (*http://iccvam.niehs.nih. gov/methods/milestones.US.htm*).

By contrast, the 2007 National Research Council (NRC) Report, *Toxicity Testing in the 21st Century; A Vision and A Strategy*, envisions a paradigmatic change in the way risk assessment is undertaken (Figure 1 outlines a possible approach to implementing the NAS vision). The study

leading to the report was sponsored by the US Environmental Protection Agency and resulted in part from EPA frustrations with the existing hazard identification and risk assessment system for pesticides. For example, Vicki Dellarco of the EPA (7 April 2011 presentation to CropLife America) reported that a typical safety evaluation for a pesticide would cost $5-6 million and use 4,000 rodents, 70 dogs and 80 rabbits. The NRC report has now sparked a major campaign within the scientific community to advance the science of toxicity testing and to put it on a trajectory that will generate better data on the potential risks to humans posed by exposure to environmental agents and result in much quicker and more efficient (and cost-effective) approaches to the testing of chemicals.

Current Activities

The response to the NRC report has been, in the main, favourable. At the 2011 Society of Toxicology meeting, the presentations were given to an overflowing meeting room whereas, a year earlier, the audience at the SOT meeting was much more modest. Numerous publications, in addition to the original report, have appeared in the toxicological literature with perhaps the most significant being a series of articles in *Toxicological Sciences* giving reactions to the report from a range of perspectives. However, the most significant developments have probably been the various 'memoranda of understanding' (MoU) signed by different US government agencies. In 2008, the EPA (ToxCast), National Institutes of Health Chemical Genomics Center (NCGC), and the National Institute of Environmental Health Sciences (NIEHS) signed an MoU to pursue a high-throughput testing strategy to set priorities for chemical selection for testing (Collins et al., 2008). A feature of this agreement was the emphasis on high-throughput test development and use to generate large amounts of data that could be subjected to new and more powerful prediction algorithms for risk assessment. Two years later, the Food and Drug Administration signed on as well.
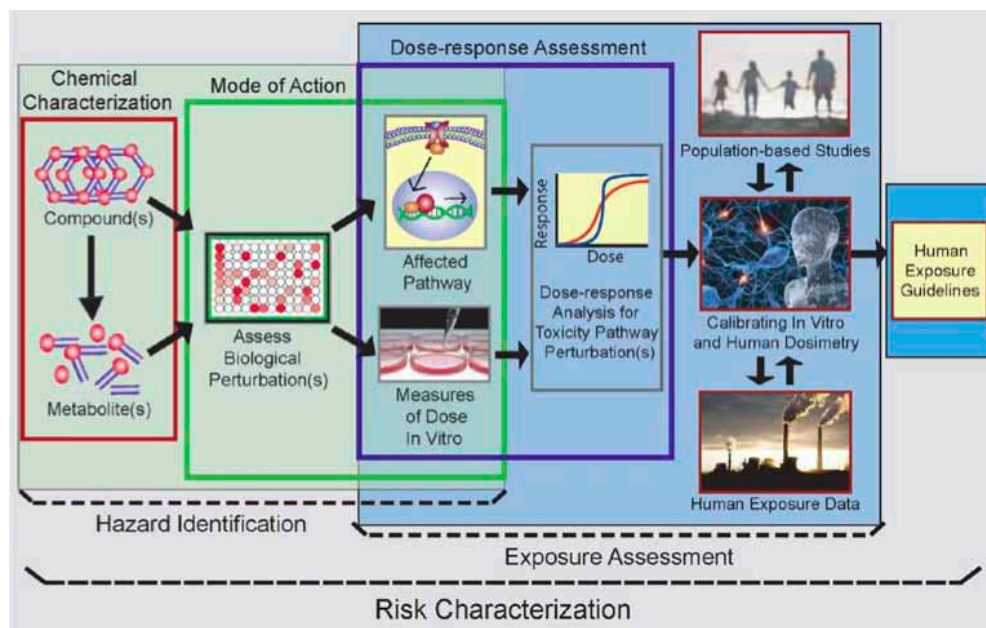

Figure 1. Risk/safety assessment following *Toxicity Testing in the 21st Century*[1]. The mapping is done against the four key components of the contemporary risk assessment paradigm: hazard identification, dose-response assessment, exposure assessment, and the overall process of risk characterisation (reprinted with permission from Krewski and Andersen, 2010[4]).

Table 2. EPA activities: a timeline for chemical testing and assay use.

Year	Partners	# Chemicals	# Assays	Comments
2005	EPA Toxcast	>100	50+	Start of programme at EPA, NRC expert panel starts project
2008	EPA Toxcast, NTP, NCGC	309	200+	Environmental chemicals, some drugs, some industrial chemicals
2010	EPA Toxcast, NTP, NCGC, FDA, NLM	1,000	500+	Dataset storage/ processing needs, chemical storage & handling increase dramatically NexGen Initiative launched
2012	EPA Toxcast, NTP, NCGC, FDA, EU, Corporations?	10,000	1,000+	Dataflow now high but due to increase

A stronger scientific foundation offers the prospect of improved risk-based regulatory decisions and greater public confidence in the decisions. The technology needed for this transformation is already available, in advanced development, or understood conceptually. The primary requisites to make this transformation are political willpower and the co-ordination of substantial public and private resources that are already allocated to hazard identification and risk assessment. The goal is clear, i.e., the replacement of current animal toxicity testing by mechanistic testing of cellular-response pathways within a cheaper and more rapid human health risk assessment framework that can handle a much larger number of chemicals than the current animal-based methods. The NRC report estimated that the full conversion of the current system to a toxicity pathway paradigm would require 10- to 20-years with an investment of the order of a billion dollars. The committee emphasised that a public-private consortium would provide the greatest opportunity for stakeholder involvement to insure more rapid acceptance of these modern approaches for testing and human health risk assessment.

The EPA is already proceeding to empower and incorporate elements of the proposed new approach (Table 2 outlines the timeline for EPA actions that expand on the standard hazard identification and risk evaluation paradigm).

The NexGen goal is to advance risk assessment by incorporating the advances in molecular and systems biology to ensure that health assessment decisions can be made in a cheaper, faster and more

robust way (S. Barone, EPA Regional Risk Assessors Conference, 9 June 2010). In particular, NexGen seeks to provide risk managers with the opportunity to 'test drive' the new assessment approaches as well as seeking input from relevant stakeholders on how they can participate in this transformation.

The Human Toxicology Project Consortium

The Humane Society family of organisations (i.e., HSI, HSUS and Humane Society Legislative Fund), following the NRC vision, has proposed the establishment of a new 'big biology' project to guide the transformation of current toxicity testing. While details remain to be determined, this multi-stakeholder effort, referred to as the Human Toxicology Project (HTP), consists of collaborators from North America and Europe. This initiative, though emphasising its toxicology application, cuts across environmental agent risk assessment, drug safety assessment, and drug development, focusing broadly on creating an understanding of perturbation of human signaling pathways in relation to toxicity and disease. Toxicity testing faces significant pressures today with the Registration, Evaluation, and Authorisation and Restriction of Chemicals (REACH) regulation in Europe and with the proposed revision of the Toxic Substances Control Act (TSCA) and other legislative initiatives in the US. Modern approaches to toxicity testing and risk assessment need to be brought online as soon as possible to support the new regulatory programmes for protecting and improving public health.

The NRC report discusses approaches for deliberate implementation over the coming 10-20 year period with emphasis on filling out a list of toxicity pathways as quickly as possible. The Hamner Institutes of Health Sciences Programme in Chemical Safety Sciences has suggested an alternative approach to accelerate implementation. The Hamner proposes to work on a series of 10 or so prototypes to evaluate the steps required to use toxicity pathway assay results for human health risk assessment. These steps include:

(1) mapping and modelling the cellular circuitry controlling each of the toxicity pathways
(2) developing appropriate, well-designed pathway assays
(3) examining relationships between pathway perturbations and adverse responses
(4) providing modeling tools to interpret the relationship of test-tube concentrations with those expected in people exposed to environmental levels of test compounds, and
(5) integrating results from these various studies to provide human health risk assessments.

The results with the group of prototypes would guide the first-generation risk assessments with the new toxicity testing paradigm, while refining the experimental and computational tools required for routine use in the future.

Why is a Consortium Needed?

While there is considerable interest in the new toxicology tools being developed, there is, as yet, also considerable duplication of effort. In order to bring the NRC vision (2007) to fruition, both funding and coordination will be required. We believe that the vision will eventually be implemented but, without some initiative like the proposed Human Toxicology Project, it could take a lot longer (and a lot more money) than would be required if an internationally co-ordinated project were established—akin to the Human Genome Project. The analogy highlights the importance of developing an urgent focus around a common mission to overcome what are mostly challenges entailing technological improvements. It also stresses the need for international leadership.

The Human Genome Project launched in 1990 after almost ten years of discussions and arguments about its appropriateness and its promise. At the time of its launch, it cost $10 to sequence one DNA base pair. Today, authorities are talking about sequencing an entire human genome (around 3 billion base pairs) for $1,000 and the current cost is in the $5-10,000 range—a technical improvement of around 5-million fold. Collins (2003) wrote about the lessons for running large biology projects including the importance of:

- Building the best teams
- Ensuring that the process is science-driven
- Meeting the managerial challenges

- Seeking international participation
- Establishing explicit milestones and quality assessments
- Striving for technological advancement that can accelerate the project
- Releasing data rapidly to demonstrate the project's value to the community
- Addressing social consequences as part of the project.

Many of these lessons speak to the cross-cutting issue of strategic planning, as well as other themes including communication and coordination. The Human Toxicology Project Consortium believes that a Human Genome Project-type effort should be marshalled to realise the NRC vision—and has called this initiative the Human Toxicology Project (HTP).

In November of 2010, a workshop was convened in Washington to examine the prospects for launching a successful HTP. The themes and recommendations that emerged from this workshop included the need to establish an Implementation Group that would, as a central challenge, hasten the development and application of new tools and approaches beyond screening and priority-setting, to hazard identification and dose-response analysis—two key components of risk assessment. Similarly, the group should also expedite the use of the new methods to diverse risk contexts (NRC, 2007), not all of which demand assays that are high-throughput. In these ways, decision-makers can use the next generation tools and approaches to make more informed and efficient responses to diverse public health concerns faced by regulators, industry,

and the public (NRC, 2007). To that end, it is important that risk assessment practitioners, regulatory scientists and science policy experts become engaged in working to advance the vision. This multi-sector diversity is a feature of the ILSI Health and Environmental Sciences Institute (HESI) Risk21 project (*hesiglobal. org/i4a/pages/index.cfm?pageid=3492*), in which the elements of the NRC vision for toxicity testing in the 21st century have been integrated into the project objective to create a systematic approach for incorporating novel approaches and technologies, as available and when appropriate, to aid in advancing human health assessments.

Implementation of the NRC vision should be seen as an iterative, progressive transformation—not an all-or-nothing switch that will take place 20 years from now. In this context, stakeholders should be attuned to opportunities to ensure incorporation of '21st century toxicology' into regulatory decisions that are on the horizon. For example, efforts are underway to revise TSCA in the US and biocides regulation in the EU. The introduction of the new tools and data derived from this methodology could be accelerated by supportive language placed in revisions to these pieces of legislation. A recent paper (Locke & Myers, 2010) discussed the challenges and opportunities of implementing the NRC vision and strategy for toxicity testing that will arise under the key provisions of TSCA, concluding that TSCA, as currently written, creates a sufficient legal foundation for the NRC vision.

In summary, the November 2010 Human Toxicology Project Consortium workshop illustrated the diversity of projects underway that are advancing pathway-based approaches to toxicity testing, which underpin the NRC vision for toxicity testing in the 21st century. Workshop participants made several recommendations for accelerating the implementation of this vision, including establishing a steering group to aid in the strategic planning, co-ordination, and communication regarding the way forward. Efforts will also be needed to commence more direct research project-based approaches to implementing the NRC vision, as well as ensure sufficient funding for the necessary work. Policy-oriented stakeholders should be attentive to opportunities to incorporate NRC vision-friendly provisions into new or amended public policies.

Conclusion

Imagine if we could progress from the current cost of $1·5 million over 3-5 years for a comprehensive toxicity screen and risk assessment for a chemical to a technology in which it would cost $1 per chemical for a complete toxicology screen and take only 1-2 weeks. This type of increase in speed and decrease in cost is what the proposed Human Toxicology Project aims to accomplish. The high-throughput systems currently being used in the programme of work undertaken by the US Tox21 collaboration have the capacity to deliver relevant data at a speed that is 1- to 10-million-times faster than current animal tests. As our understanding of pathways increases and

the development of new more relevant tests accelerates, this data flood will allow both much faster and also more relevant and precise risk assessment. The Human Genome Project and the enormous advances in technical speed and relevance are an excellent analogy for what will happen as we implement the NRC 2007 vision.

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Human Skin Disease Models

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Background & Objectives

Reconstructed human epidermis (RHE) built up from differentiating keratinocytes, and reconstructed full-thickness skin (RHS) comprised of an additional dermis equivalent represented by fibroblasts embedded into a collagen matrix, have become available from commercial sources (e.g., EpiDerm, SkinEthik; RHE only: EPISKIN; RHS only: Phenion-FT) in high quality (Van Gele et al., 2011). The Organisation for Economic Co-operation and Development (OECD, 2011) has adopted protocols for testing skin corrosivity (Guideline 431), skin irritancy (Guideline 439), and phototoxicity (in case of potentially non-penetrant UV sensitisers; Guideline 432) in RHE (Netzlaff et al., 2005; Kanderova et al., 2009) and stated that reconstructed tissue can be used, too, given results comparable to those obtained by testing in human skin *ex vivo* are to be expected (Guideline 428/Guidance Document 28). This, in fact, appears to hold true as shown in a German Federal Ministry of Education and Research (BMBF)-funded German multi-lab study investigating nine test agents enabling estimation of uptake in humans. Permeability of RHE is higher than of human skin *ex vivo*, yet there appears to be a well-defined ratio (Schäfer-Korting et al., 2006; 2008). The predictive power of data derived from studies in excised human skin for *in vivo* bioequivalence has been reported (Franz et al., 2009).

A topic long neglected is biotransformation of compounds in the skin (Oesch et al., 2007), which can be the first step in sensitisation and genotoxicity, but is also relevant for skin penetration of the native drug as shown for a glucocorticoid diester used in, e.g., dermatitis (Lombardi Borgia et al., 2008). Thus local biotransformation in the skin may also influence efficacy of the treatment. A comparison of biotransformation in freshly excised human skin and RHS is subject of current research (BMBF funding).

In addition to commercially available constructs, in-house models of human skin are used,

in particular for fundamental research. Constructs including specialised cell types of the skin are needed to address specific questions, e.g., melanocyte-enriched RHE can be used for a study of UV-induced effects on the epidermis. Engineering of skin models of increasing complexity is based on increasing insight into intense cross-talk between cells related to cell type and differentiation status. Skin explants providing the full spectrum of cells embedded in human skin may be an alternative (Lebonvallet et al., 2010), yet availability of suitable material is limited.

Aiming for improved preclinical testing while avoiding animal experimentation, there is also a clear need for disease models based on organotypic constructs derived from human cells in general. This also holds true for skin diseases, since many of those (e.g., severe types of ichthyosis, chronic dermatitis, skin ulcers, skin cancer) are notoriously poorly responsive to current treatment options.

Current Research

Ongoing research in the engineering of diseased skin and the use of constructs for science has been reviewed recently (Carlson et al., 2008; Semlin et al., 2011). At the Freie Universität Berlin, scientists aim for the establishment of disease models for a broad spectrum of skin diseases, and make use of various approaches.
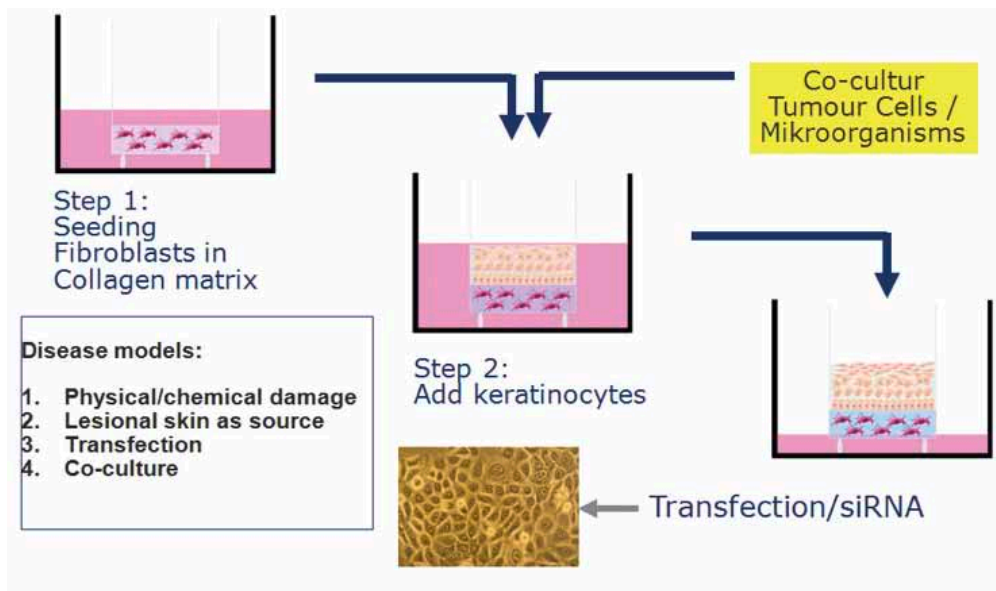

Figure 1. Schematic presentation of the engineering of human skin constructs in vitro, both of normal skin and when aiming for skin disease models. Approaches to skin disease comprise (1) damage of the construct, (2) growing the skin from cells derived from lesional skin, (3) knock-down of disease associated genes, and (4) co-culture with micro-organisms and tumour cells, respectively.

Physical / Chemical Damage

A first approach (Figure 1) consists of damaging the skin surface. Physical damage by tape stripping is frequently used for *in vivo* studies for skin penetration. The process needs to be strictly controlled and the amount of horny layer removed has to be quantified for each tape (Lademann et al., 2009; Zhai et al., 2007). Abrasion has been used for *ex vivo* studies, e.g., for the study of systemic availability of metal powders. Cobalt and nickel uptake appears to increase dramatically when applied to damaged skin *ex vivo*, which however does not hold true with chrome (Filon et al., 2009).

For modelling of atopic dermatitis, application of tumour necrosis factor-α (TNFα) appears to be an interesting option since this cytokine is released from keratinocytes, e.g., due to patient´s scratching and induces the disease cascade of lesional skin. A relevant increase in interleukin-6 release is obtained when applying TNFα to the surface of RHS, which can be suppressed by topical glucocorticoid treatment (Weindl et al., 2011). This may offer an opportunity for avoiding animal experiments in preclinical drug development, both with respect to the pharmaceutically active agent and when searching for the most suitable carrier as indicated by our various investigations of nanocarriers (NanoDerm project; EFRE funding).

Aiming for the topical use of morphine-loaded nanocarriers (EFRE funding) for the severe pain at the donor site of skin grafts or in skin ulcers (Vernassiere et al., 2005), we also had to investigate whether the opioid may interfere with wound healing. Besides using the scratch test in the monolayer culture, we aimed for extended research into the organotypic models. Carbon dioxide laser irradiation allowed us to generate well-defined wounds in RHS, and morphine as well as solid nanoparticles used for opioid loading enhanced re-epithelisation of the wound ground by stimulation of keratinocyte proliferation and migration (Küchler et al., 2010).

Lesional Cells & Gene Knock-Down

Another approach can consist of the engineering of skin models using cells from lesional skin (Figure 1), given that there is sufficient material available. Alternative approaches also need to be considered, and transgenic cells might be the solution. When investigating atopic dermatitis, deficiency in the filament aggregating protein filaggrin (FLG) is seen in up to 60% of the patients, which is related to single or double-allele mutations of the FLG gene (Elias et al., 2009). Thus it appeared reasonable to study the effects of FLG knock-down by siRNA, which became possible by the work of two independent research groups (Mildner et al., 2010; Küchler et al., in press). In accordance with lesional skin in atopic dermatitis, histology of RHS grown from keratinocytes following FLG knock-down reflects eczematous skin and the construct is more susceptible to water and sodium dodecylsulfate exposure as to be derived from enhanced interleukin release

compared to controls. Most interestingly, the disease model appears to be highly permeable for lipophilic agents, too (Küchler et al., in press). Permeability of lesional skin in atopic dermatitis exceeding permeability of healthy skin and thus explaining the specific 'sensitivity' of atopic patients is a matter of ongoing clinical research (Angelova-Fischer et al., 2011). This underlines the potential of disease models for future research in pathology.

Both siRNA knock-down of another disease-related gene in normal human keratinocytes and growing RHS using lesional keratinocytes have been used to build up congenital ichthyosis skin, which is due to corneodesmosin (CDSN) loss (Oji et al., 2010), in addition to FLG mutations as second cause (Elias & Schmuth, 2009). The constructs have been characterised in full including the permeability for hydrophilic and lipophilic compounds, both being enhanced over the permeability of constructs grown from normal human keratinocytes while avoiding *CDSN* knock-down (Oji et al., 2010). Since efficient therapy for this very severe disease does not exist, this construct offers the possibility to start drug research from scratch on.

Co-Cultures

Organotypic models both for fungal infections of mucous membranes and skin as well as for melanoma and non-melanoma skin tumour have been set up and used to investigate drug effects (Semlin et al., 2011). Using a non-melanoma skin

tumour model first described by Alt-Holland (2008), efficacy of photodynamic therapy has been demonstrated by Hoeller Obrigkeit and coworkers (2009), and the model is used for the testing of new anticancer drugs (Schwanke et al., 2010) and their formulations as well (research in the NanoDerm and NanoSkin projects).

Yet, a further improvement is advocated, as the competence of the immune system is of ultimate importance in the removal of both infectious agents and tumour cells, and it is of importance for atopic dermatitis, too. Embedding of immunocompetent dendritic cells is subject of intense research (Dezutter-Dambuyant et al., 2006; Facy et al., 2005). A limited survival time of primary dendritic cells and the activity of respective cell lines raise problems, which need to be overcome. As this becomes possible, it is anticipated that the skin sensitisation area will profit from these constructs, too.

In summary, great progress has been obtained with the reduction or even replacement (phototoxicity of chemicals in the EU) of animal testing by using reconstructed human epidermis. The availability of human-based material allowing for the cross-talk of keratinocytes and fibroblasts, as well as the ongoing research in skin disease models, offers new approaches for future reduction of animal testing in preclinical drug development. Hopefully, the progress obtained in the field of reconstruction of diseased skin may be a role model in the set-up for models of diseases for other organs. This offers new opportunities to control the

numbers of laboratory animals needed. In Germany, the numbers are currently increasing steadily, and biomedical/drug research makes up for more than 50% of laboratory animal use (BMELV).

Acknowledgements

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The Virtual Liver
Spatial·Temporal Modelling of Tissue Damage & Regeneration

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Background & Objectives

There is currently limited knowledge on how cells behave in a coordinated fashion to establish functional tissue architecture and to respond to chemically induced tissue damage during regeneration. A vision of this project is that the spatial·temporal events during tissue damage and regeneration can be simulated *in silico*. The Virtual Liver, together with its co·operating projects DETECTIVE, NOTOX, ESNATS, CancerSys and ToxGenom, focuses on the establishment of a three·dimensional model of the liver that correctly recapitulates alterations of the complex microarchitecture, both in response to, and during regeneration from, chemically induced liver damage. Since the exact position and metabolic capacity of the individual cells of the model are known, it should also be possible to simulate to what degree a certain pattern of chemically induced liver damage compromises the metabolic capacity at the organ level. Finally, a long·term goal will be to integrate intracellular mechanisms into each cell of the model that generate single cell f≠a long·term goal, as many of the critical intracellular key mechanisms still need to be elucidated.

Deliverables & Milestones Achieved During 2010

Simulation of Tissue Destruction & Regeneration by Spatial-Temporal Modelling

A key achievement in 2010 was the establishment of a model that correctly recapitulates the complex spatial·temporal events after induction of hepatic tissue damage (Figure 1; Hoehme et al., 2010). The model includes a liver lobule, the smallest functional unit of the liver. Although the primary achievement of the model is the possibility to

quantitatively predict aspects of tissue toxicity, such as spatial-temporal patterns of replacement proliferation or alterations of tissue architecture, the results of the simulations can also be visualised as a movie. The example in Figure 1 shows a ten-day process after intoxication with CCl$_4$, a model compound that induces pericentral liver damage and is prototypical for many other hepatotoxic chemicals that are activated by cytochrome P450 enzymes predominantly expressed by pericentral hepatocytes. Upon induction of liver damage, some of the surviving hepatocytes proliferate. This results in a transiently disordered tissue architecture. However, over a 10-day process the initial microarchitecture characterised by columns of hepatocytes along sinusoids is re-established (Figure 1). The result of the mathematical simulation can also be visualised as a movie, which is accessible online at *ifado.de/forschung_praxis/ projektgruppen/susceptibility/forschung/ link2/index.html.*

Such spatial-temporal models are established in a two-step process. First, the tissue architecture has to be reconstructed, which is achieved by confocal laser scans of liver tissue (Figure 2). This leads to a reconstructed liver lobule, where the position of each individual cell in a 3D space is known. In addition, the architectural principles, such as bifurcation characteristics, size and density of the microvessel network are included. This 'static reconstruction' serves a basis for further spatial-temporal modelling. The smallest unit of such models is the individual cell that can divide leading to an increased space requirement. The exact behaviour of the cells in the model is controlled by so-called 'process parameters'. Process parameters are experimentally determined measures, such as the probability of cell division or cell death at a given position of the lobule at a given time, polar cell adhesion of hepatocytes, micromotility and cell cycle parameters. Integration of the process parameters and of a so far unrecognised tissue organisation principle (hepatocyte sinusoidal alignment, described later) finally allowed the correct modelling of the situation after tissue destruction (Figure 1). Besides their use in systems biology, the novel techniques are also widely applicable in other fields of research, e.g., for analysis of the impact of genetic

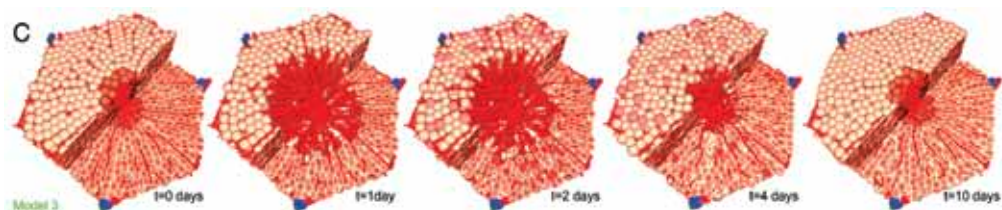
Figure 1. Spatial-temporal model of a liver lobule showing a ten-day process after intoxication with CCl4 as a prototypical compound for chemicals causing pericentral liver damage. The model has been established using male C57Bl6/N mice but can be extrapolated to the human situation (Hoehme et al., 2010).
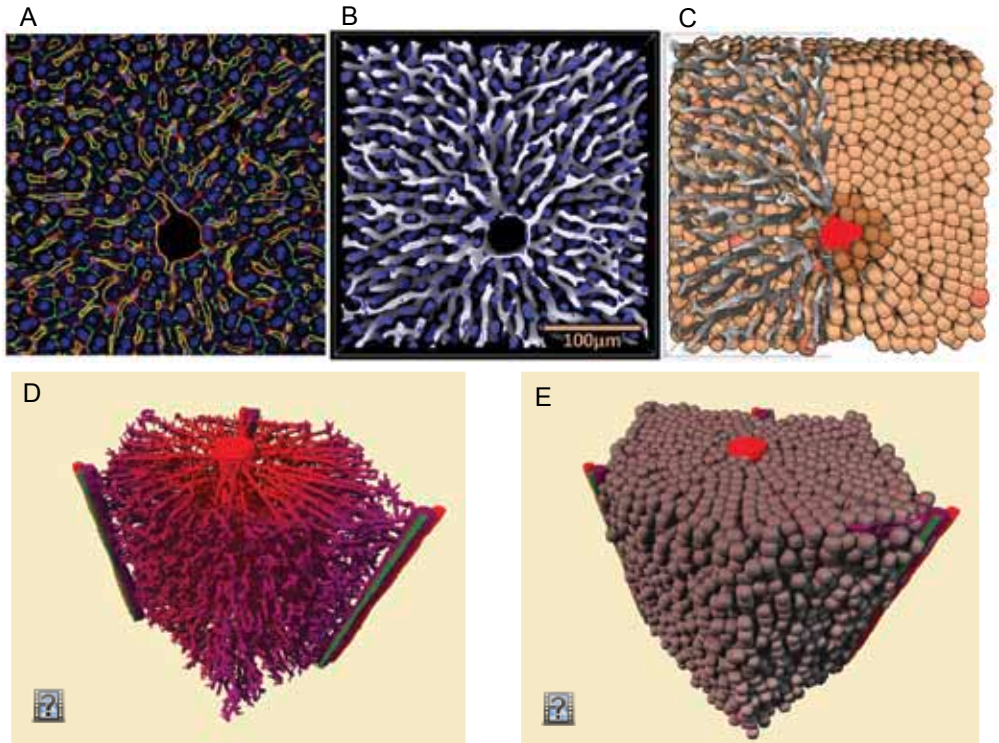
Figure 2. Tissue reconstruction as a precondition for spatial-temporal modelling. Confocal Z-scans (A) are used to reconstruct the microvessel network (B) and small tissue blocks (C). D-E: Several segments are composed to reconstruct entire liver lobules (from: Hoehme et al., 2010).

modifications on tissue architecture of mice (Braeuning et al., 2010).

Prediction of a Novel Key Mechanism of Liver Regeneration

One of the achievements of the novel approach is that tissue architecture (e.g., calculated as the contact surface between hepatocytes and sinusoids) can be analysed by quantitative measures. This provides the opportunity to (i) simulate the scenario after tissue damage and (ii) experimentally determine the situation to analyse if the experiment and the model prediction are in agreement. Interestingly,

experimental data and simulation were only in agreement when a so far unrecognised mechanism was assumed, which we named "hepatocyte sinusoidal alignment" (HSA). HSA means that during hepatocyte division the orientation of the mitotic spindle is initially random (Figure 3A). However, after mitosis the daughter cells rapidly realign in the direction of the closest sinusoid. This mechanism was predicted by the model and could later be validated by extensive tissue reconstructions, where an automated algorithm measured the angles between the lines through the centre of two daughter hepatocytes and the orientation

Figure 3. A. Hepatocyte sinusoidal alignment (HAS). During cell division the orientation of the mitotic spindle is random but after mitosis the daughter cells realign in the direction of the closest sinusoid. HSA is a 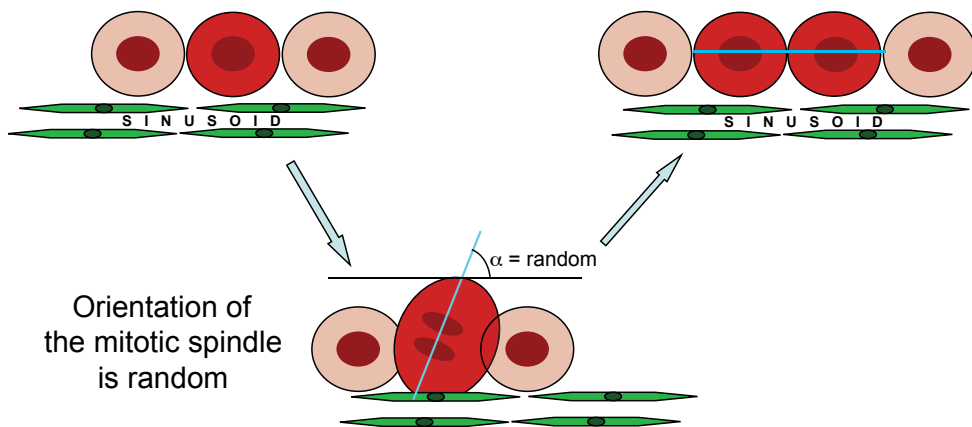previously unrecognised key mechanism, which is essential to restore functional microarchitecture after chemically induced liver damage.

of the closest sinusoid (Figure 3B). Whether compromised HSA contributes to liver toxicity and/or liver diseases is currently under investigation.

Integrating Intracellular Mechanisms

A future goal of the Virtual Liver is to integrate intracellular mechanisms, such as signalling networks and control systems of RNA expression, into the individual cells of the model. The models of intracellular networks will generate single cell fate decisions whose influence on tissue architecture can then be simulated. This is a long-term goal as the intracellular processes of decision-making are currently far from being understood. It is clear that the relationship between intracellular mechanisms and cell fate decisions can be studied more easily *in vitro*. For this purpose hepatocyte *in vitro* systems are needed that closely reflect the *in vivo* situation. An important

Figure 3. B. Experimental validation of HAS. Reconstruction of the microvessel (sinusoidal) network of liver tissue during regeneration after intoxication with CCl4. BrdU incorporation is visualized by red fluorescence. The green lines connect the centres of BrdU positive daughter cells. The blue lines visualise the orientation of the closest sinusoid. The analysis showed that the angle of the blue and green lines is already small shortly after cell division, indicating that daughter hepatocytes align themselves in the direction of sinusoids (from Hoehme et al., 2010).
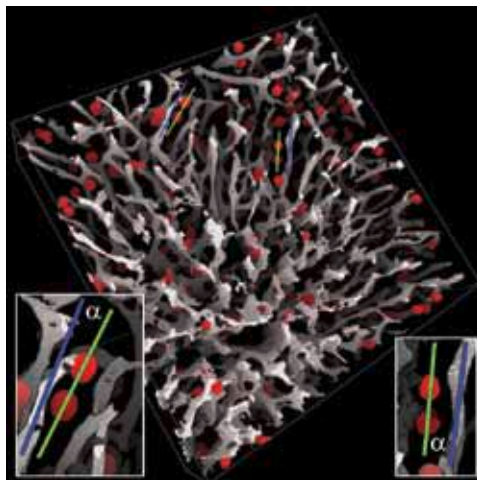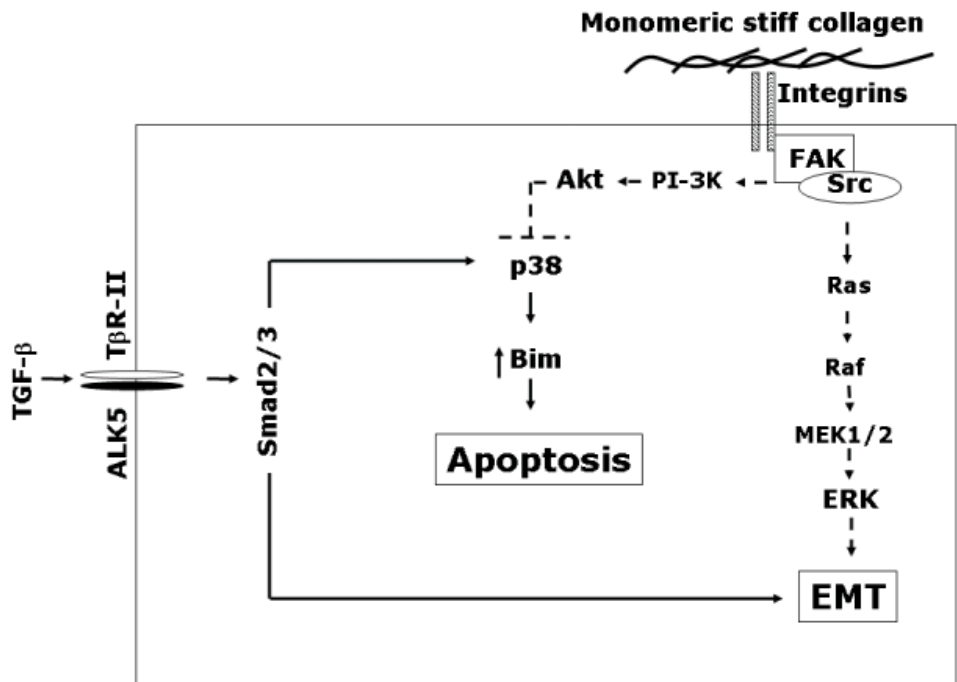
Figure 4. Mechanisms of how hepatocyte function is altered as a consequence of cell isolation from the liver and cultivation in vitro. Focal adhesion kinase (FAK) is activated as a consequence of hepatocyte attachment to the matrix of the culture dish. As a consequence Ras/Mek/Erk signalling triggers features of epithelial-to-mesenchymal transition (EMT) commonly considered as 'dedifferentiation'. Furthermore, activation of PI-3K/AKT leads to apoptosis resistance.

milestone achieved in 2010 was the elucidation of the molecular mechanisms explaining why cultivated hepatocytes differ from hepatocytes *in vivo* (Godoy et al., 2009; Zellmer et al., 2010). Briefly, the attachment of hepatocytes to the matrix of culture dishes activates focal adhesion kinase (FAK) which induces two processes: (i) activation of the Ras/Mek/Erk pathway which triggers features of epithelial to mesenchymal transition, generally known as "dedifferentiation", (ii) activation of PI3K/Akt which leads to inhibition of p38 and apoptosis resistance (Figure 4). The latter finding is in contrast to the common view that

hepatocyte dedifferentiation *in vitro* is a passive process representing a "loss of function" phenotype. The explanation that hepatocyte dedifferentiation is driven by overactive signalling has paved the way to better *in vitro* systems as it is technically easier to decrease overactive pathways then substitute for missing mechanisms.

The novel hepatocyte *in vitro* systems led to substantial progress concerning the agreement between *in vivo* and *in vitro* data. Previously, it has been reported that there is minimal or no overlap between chemically induced gene expression alterations in cultivated hepatocytes and

A

Rats
in vivo
1299

2 (3)   18 (25)

(2)

Rat
hepatocytes
standard

Rat
hepatocytes
modified

98 (10)

253   1136

from: Kienhuis et al., 2009

B

Stress response
associated genes

P=3.18×10⁻⁴

In vivo
fold change (log2)

In vitro
fold change (log2)

C

Proliferation
associated genes

P=0.286

In vivo
fold change (log2)

In vitro
fold change (log2)
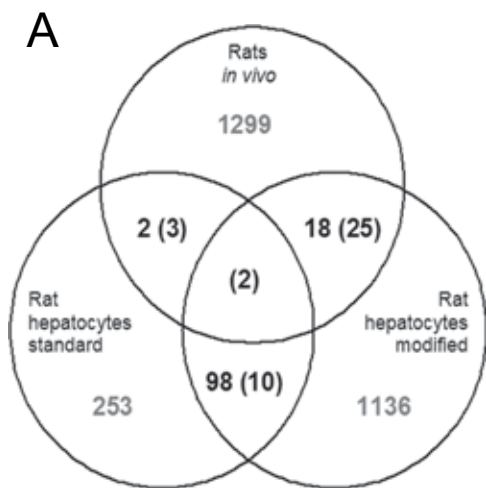
Figure 5. A. Minimal or no overlap between gene expression alterations induced by par-acetamol in rat liver *in vivo* and in cultivated hepatocytes cultured in a standard or in a modified culture medium as reported in a previous study (Kienhuis et al., Toxicol. Sci. 2009; 107, 544-52). The negative result is in agreement with the current sceptical view of many experts concerning cultivated hepato-cytes for toxicogenomics studies. B. Improve-ment by the current project: stress associated genes (including DNA repair and metabolism-associated genes show a good correlation *in vivo* and *in vitro*. For this experiment, genes were selected from an *in vivo* study that were up or down regulated by the liver carcinogens aflatoxin B1, 2-nitroflurene, methapyrilene and piperonylbutoxide. Cultivated hepatocytes were then exposed to the compounds and expres-sion alterations were determined by RT-qPCR. C. In contrast to the stress-associated genes (B) no *in vivo/in vitro* correlation was observed for genes associated with proliferating cells. The reason for this discrepancy is that culti-vated hepatocytes, in contrast to liver tissue *in vivo,* do not show replacement proliferation.
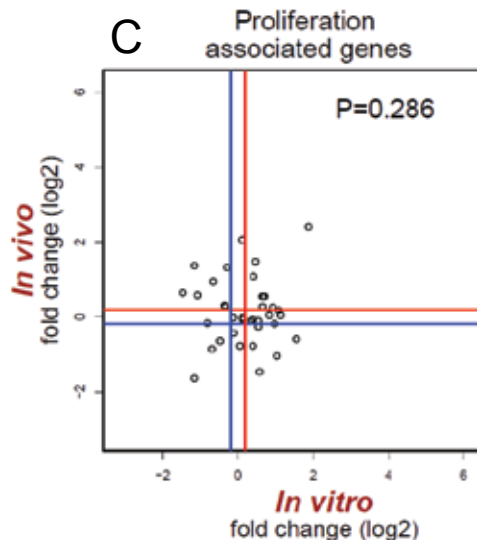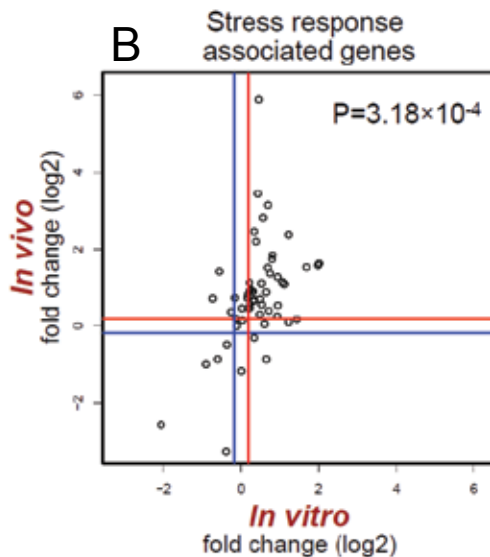
in rat liver *in vivo* (Figure 5A). However, using the *in vitro* system established in our project a significant correlation was obtained between gene expression alterations *in vivo,* in the livers of rats after oral administration of liver carcinogens, and in cultivated hepatocytes incubated with the same compounds (Figure 5B). It should be noted that genes associated with proliferating cells represent an exception of this rule and do not show an *in vitro/in vivo* correlation (Figure 5C). The reason for this discrepancy is that primary hepatocytes cultivated under the current conditions can not undergo replacement proliferation. While a hepatocyte *in vitro* system capable of replacement proliferation would represent further progress, its deficiency does not necessarily compromise the practical applicability of the current system. It should be taken into account that replacement proliferation represents a secondary event after toxic stress to cells or tissues and the primary events may already be sufficient to capture a compound specific fingerprint at the gene expression level.

Challenges & Next Steps

The project faces two major challenges, namely 'vertical integration' and improvement of the applicability of the established models for pharmaceutical industry and toxicology. Vertical integration means that the current model (Figure 1) will be linked to the organ and to the sub-cellular level. For this purpose, model predictions of how certain tissue destruction patterns compromise metabolic organ function, and decrease elimination of drugs, have already been made and will be experimentally validated in 2011/2012. Algorithms based on RNA expression patterns to identify certain classes of hepatotoxic compounds (e.g., non-genotoxic carcinogens) are of high interest for pharmaceutical industry and toxicology. Currently, a large classification study using the novel *in vitro* system is ongoing; algorithms are established in training sets of compounds and confirmed in independent sets of follow-up compounds. The results will be available in the first half of 2012.

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The IMI eTOX Project
Efforts to Develop *In Silico* Tools to Predict *In Vivo* Toxicity

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Background

The eTOX project was proposed under the first call of the Innovative Medicines Initiative (IMI; *imi.europa.eu*) under the title: 'Integrating bioinformatics and chemoinformatics approaches for the development of expert systems allowing the *in silico* prediction of toxicities'.

IMI is Europe's largest public-private initiative aiming to speed up the development of better and safer medicines for patients. IMI supports collaborative research projects and builds networks of industrial and academic experts in order to boost pharmaceutical innovation in Europe. IMI is a joint undertaking between the European Union and the pharmaceutical industry association EFPIA. IMI supports research projects in the areas of safety and efficacy, knowledge management and education and training. Under the pillar 'safety', eTOX was proposed because the need for improved toxicological databases and prediction tools was identified a bottleneck during early drug development.

During these phases, *in vivo* studies are often unveiling side effects that presently cannot be predicted from the chemistry. However, mechanisms such as cross-target activation or inhibition, or straight chemistry-linked toxicities are amenable to prediction. Early *in silico* prediction of such cases would increase the quality of drug candidates and ensure a lower attrition rate before and during the first GLP animal studies. This could also reduce the number of animals (3Rs) used in preclinical studies necessary to select drug.

Therefore, the eTOX project aims to build a toxicology database relevant to pharmaceutical development and to elaborate innovative methodological strategies and novel software tools to better predict the toxicological profiles of new chemical entities in early stages of the drug development pipeline based on existing *in vivo* study results. This is planned

to be achieved by sharing and jointly exploiting legacy reports of toxicological studies from participating pharmaceutical companies. The project coordinates the efforts of specialists from industry and academia in the wide scope of disciplines that are required for reliable modelling of the complex relationships existing between molecular and in vitro information, and the *in vivo* toxicity outcomes of drugs.

eTOX began on 1 January 2010. The project is carried out by a consortium comprising 25 organisations (13 pharmaceutical companies, 7 academic groups and 5 SMEs) with complementary expertise. With €5 million from the European Union, the total budget of the project is €13 million, which will fund this initiative during five years.

Objectives

The key objectives of eTOX are the collection of toxicological data from proprietary (EFPIA) and public sources, the construction of a common toxicological database from which data can be mined and extracted to build expert system or predictive models for *in vivo* toxicity.
The integration of these different objectives into a strategy is depicted in Figure 1.

The proposed strategy includes the integration of innovative approaches in the following areas:

- Data sharing of previously inaccessible high-quality data from toxicity legacy reports of the participating pharmaceutical companies

- Database building and management, including procedures and tools for protecting sensitive data
- Ontologies and text mining techniques, with the purpose of facilitating knowledge extraction from and efficient usage of legacy preclinical reports and biomedical literature
- Physico-chemical and structure-based approaches for the molecular description of the studied compounds, as well as of their interactions with the anti-targets responsible for secondary pharmacologies
- Prediction of DMPK (drug metabolism and pharmacokinetic) properties and incorporation of physiologically-based pharmacokinetic (PBPK) predictions
- Bioinformatics and systems biology approaches in order to cope with the complex biological mechanisms that govern *in vivo* toxicological events
- Statistical modelling tools to derive QSAR models.

The expectation is that during the last phase of the project, the partners will have a system in their hands, which will allow them to compare a new drug candidate or chemical entity with toxicological results for structurally related compounds ('read-across') and to predict its potential *in vivo* toxicity on the basis of expert systems and QSAR models. The legal framework has been developed which prepares the access of the successful system to the broader public after the end of the project.

Deliverables

The eTOX has a series of ambitious

deliverables, which can only be achieved by a seamless integration of the complementary expertise of all partners of the project:

• Setup of a toxicological database with high quality *in vivo* data (e.g., from systemic toxicity studies estimated in the range of 10,000 reports)

and secondary pharmacology *in vitro* assays. This repository will form the basis of prediction model development

• Critical assessment of the diverse approaches towards *in silico* toxicology and their value in drug discovery and development

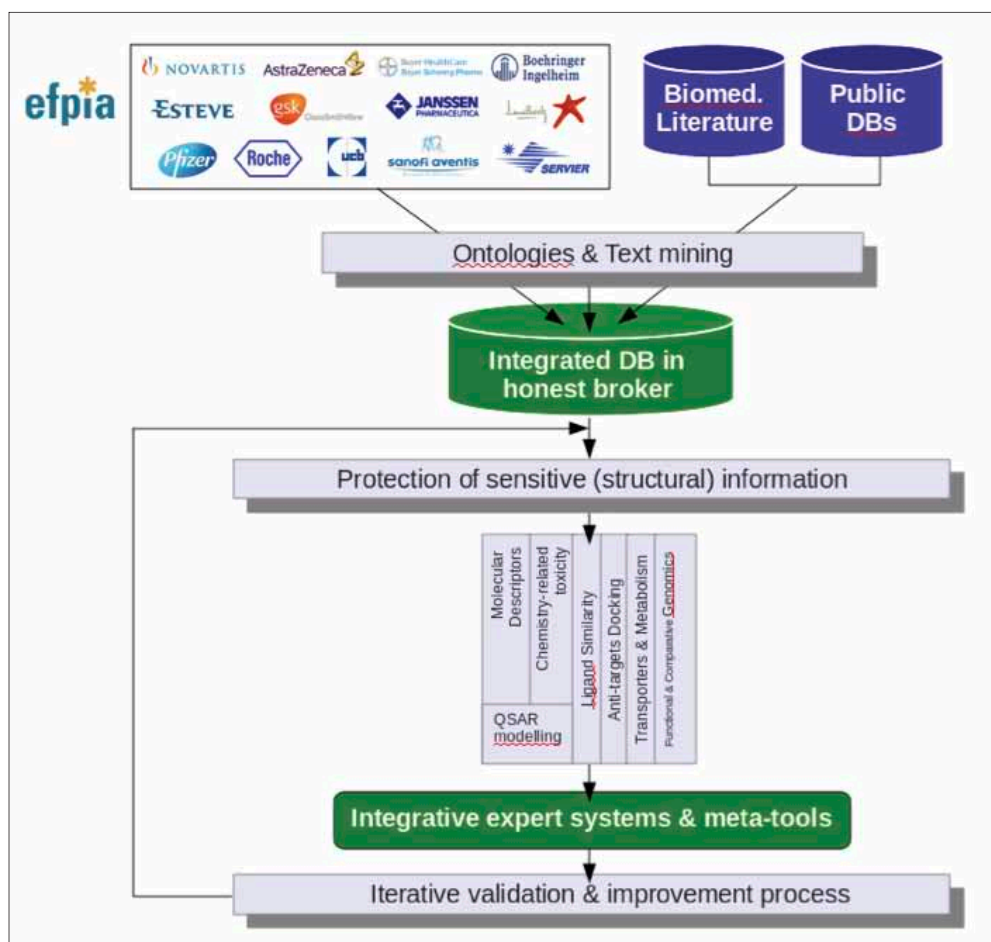• Construction of prediction models

Figure 1. The eTOX project collects toxicological data from pharmaceutical (EFPIA) companies and public sources, incorporates it into a database hosted by a honest broker to safeguard IP issues related to these data. The database will then serve as source for mining and construction of *in silico* tools to predict *in vivo* toxicity.

for selected *in vivo* endpoints to identify compound liability for target organ toxicity and to identify the primary sensitive target organ(s) after systemic exposure.

The deliverables were subdivided into goals for the different projects phases. The achievements of the first project phase covering slightly more than the first year of project duration are provided in the following chapter.

Achievements & Next Steps

The first and main hurdle to be overcome for the eTOX project was the initiation of safe sharing of proprietary data from the EFPIA companies. During the first phase the eTOX project achieved:

- To develop a complex framework of legal statutes and IT technical provisions to overcome the hurdles of sharing proprietary data of EFPIA companies.
- To design and successfully test strategies for the masking of sensitive structural information of compounds to enable secure exploitation of the associated data. These strategies include strategies and computational tools to prevent possible reverse engineering of confidential chemical structures while allowing relevant predictions of toxicological parameters.
- To design and specify the first version of the central database, hosted by Lhasa, that will be used to store the

shared toxicity data extracted from legacy reports of EFPIA companies.

Beyond overcoming the hurdle of safe data sharing eTOX accomplished:

- An agreement upon the modular architecture of the eTOX predictive system
- The development of first predictive modules to prototype the proposed architecture; an example for this is the multi-scale system developed to predict drug-induced LQTS and which has been recently published in J. Chem. Inf. Model. (see References)
- The successful execution of a data extraction pilot study, aimed at identifying an appropriate methodology to efficiently extract data from approximately 10,000 systemic toxicity legacy reports from the EFPIA companies for feeding the database
- The compilation and critical assessment of available public data sources useful to build and train predictive toxicity models and algorithms
- The development of a first version of a consensual, common toxicity ontology for seamless data gathering, integration and exploitation for predictive purposes.
- The set-up of a content-rich, continuously updated project website (*e-tox.net*), which incorporates an intranet section intensively populated with background information and useful resources (bibliography, databases, forums, etc.).

A series of publications related to eTOX have been generated by several project partners and are listed in the next section.

Publications

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Partners

The following list provides the names of the 13 EFPIA companies and the 12 public partners (academic institutions and SMEs):

EFPIA

- Novartis Pharma AG, Basel, Switzerland (Project Coordinator)
- AstraZeneca AB, Södertälje, Sweden
- Boehringer Ingelheim International GmbH, Ingelheim, Germany
- Bayer HealthCare, Berlin, Germany
- Laboratorios del Dr Esteve, S.A., Barcelona, Spain
- GlaxoSmithKline Research and Development LTD, Brentford, UK
- Janssen Pharmaceutica NV, Beerse, Belgium
- UCB Pharma SA, Brussels, Belgium
- H. Lundbeck A/S, Valby, Denmark
- Pfizer Limited, Sandwich, UK
- F. Hoffmann-La Roche AG, Basel, Switzerland
- Sanofi-Aventis GmbH, Frankfurt, Germany
- Les Laboratoires Servier SA, Neuilly-sur-Seine, France

Universities, Research Organisations, Public Bodies & Non-Profits

- Fundació IMIM, Barcelona, Spain
- Fundación Centro Nacional de Investigaciones Oncológicas Carlos III, Madrid, Spain
- European Molecular Biology Laboratory, Heidelberg, Germany
- Liverpool John Moores University, Liverpool, UK
- Technical University of Denmark, Kgs. Lyngby, Denmark
- Universität Wien, Vienna, Austria
- Vereniging voor christelijk hoger onderwijs, wetenschappelijk onderzoek en patiëntenzorg, Amsterdam, Nederland

SMEs

- Lhasa Limited, Leeds, UK
- Inte:Ligand GmbH, Vienna, Austria
- Molecular Networks GmbH, Erlangen, Germany
- Chemotargets SL, Barcelona, Spain
- Lead Molecular Design S.L., Sant Cugat del Vallès, Spain

Update on EPA's ToxCast Programme

Providing High-Throughput Decision Support Tools for Chemical Risk Management

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The US EPA's ToxCast research programme was launched in 2007 with the goal of evaluating the use of high-throughput bioassays to detect key biological targets and pathways that could be potential targets for chemicals and as a consequence of the interaction cause diseases such as cancer, reproductive toxicity or birth defects (Dix et al., 2007). As noted by the NRC in its vision for a new paradigm in toxicity testing (NRC, 2007), the traditional approach to toxicology uses expensive and time consuming animal-based testing approach and is inadequate to cover the large numbers of chemicals in commerce. In addition, since it does not provide mechanistic information on how the chemicals exert toxicity, there remain large uncertainties in extrapolating data across dose, species and life stages. ToxCast addresses many of the issues raised in the NRC report. It is a multi-year, multi-million dollar effort to comprehensively apply batteries of *in vitro* tests against chemicals with known toxicological phenotypes derived from traditional guideline studies for cancer, reproductive impairment and developmental disorders (see Martin et al., 2009a,b; Knudsen et al., 2009 for description and analyses of the traditional legacy databases). With a commitment to transparency and public release of all data (see *epa.gov/actor* for access), it is the most strategic and co-ordinated public sector effort to transform toxicology. The goal is to acquire sufficient information on a range of chemicals so that 'bioactivity signatures' can be discerned that identify distinctive patterns of toxic effects, or phenotypes, observed in traditional animal toxicity testing. The ToxCast™ predictive bioactivity signatures are based upon physico-chemical properties, biochemical properties from high-throughput screening (HTS) assays, cell-based phenotypic assays, genomic analyses of cells *in vitro*, and responses in non-mammalian model organisms.

Table 1. HTS components of ToxCast Phase I (see actor.epa.gov/actor/faces/ToxCastDB/ DataCollectionList.jsp for a full listing of the assays for each platform; (*) indicates data soon to be released in AcTOR)

Technology Platform Source	Description	N° Assays	Reference
ACEA	Real-time cell electronic sensing (RT-CES) of growth of A549 cells	7	No individual publication available
Apredica (formerly Cellumen)	Cellular high content screening (HCS) evaluating cellular markers such as stress pathways, mitochondrial involvement, cell cycle, cell loss, mitotic arrest & the cytoskeleton in HepG2 cells	19	No individual publication available
Attagene	Multi-plexed transcription factor profiling in HepG2 cells	81	Martin et al. (2010)
Bioseek	Elisa based readouts of interactions of co-cultures of primary human cells	174	Houck et al. (2009)
CellzDirect	qNPA on select genes relevant to xenobiotic metaobilism in primary human hepatocytes	16	Rotroff et al. (2010)
Gentronix	GreenScreen genetic toxicity assay using GADD45a GFP in TK6 cells	1	Knight et al. (2009)
NCGC	qHTS profiling of nuclear receptor function in agonist & antagonist mode by reporter genes using a variety of cell types	19	Huang et al. (2011)
NHEERL (*)	Mouse embryonic stem cell cytotoxicity & differentiation	8	Chandler et al. (2011)
NHEERL (*)	Zebrafish embryonic development assay	1	Padilla et al. (in prep)
NovaScreen	Biochemical profiling, largely using human proteins, of receptor binding, enzyme assays, GPCRs & ion channels	292	Knudsen et al. (2011)
Solidus	Cytotoxicity assay which included exogenous P450 metabolising systems in Hep3B cells	4	No individual publication available

Phase I of ToxCast involved the evaluation of 309 unique chemicals against a battery of 621 *in vitro* assays from different technology platforms, most of which now have an associated descriptive publication (Table 1).

An overview of the Phase I results (Judson et al., 2010) demonstrated a broad spectrum of chemical activity at the molecular and pathway levels, with chemicals interacting with an average of about 50 assays, and some interacting with more than 100 assays. Many expected interactions were seen in the data, including endocrine and xenobiotic metabolism enzyme activity. When assays were mapped to biological pathways, chemicals showed widely varying promiscuity across pathways, ranging from no activity to activity against dozens of pathways.

The ToxCast chemicals were largely derived from a list of food use pesticides, and hence are generally regarded as non-genotoxic chemicals. However, 21 of the ToxCast 309 chemicals were shown to induce liver tumours in chronically exposed rats. A bioactivity signature was developed that linked several biologically plausible pathway interactions to liver cancer. This bioactivity signature suggests that if a chemical interacts with the peroxisome proliferator-activated receptor gamma pathway (PPARg) and one of more of the following pathways—PPARα activation, cytokine CCL2 up-regulation, androgen antagonism, or oxidative stress—there is a significantly increased likelihood for inducing rat liver tumours when compared to non-genotoxic chemicals activating none

or only one of these processes (Judson et al., 2010). This prediction model is now being tested *in vivo* in rats by the National Toxicology Program (Michael De Vito, personal communication).

In the past year, efforts have continued to explore and interpret the Phase I results, and predictive bioactivity signature models relating the HTS activity to reproductive function, developmental toxicity and vascular development have been constructed (Table 2). These models are based on a common workflow of identifying individual ToxCast assays that correlate with *in vivo* phenotypes from particular types of mammalian toxicology studies, combining the assays into their biological pathways, and finally applying linear discriminant analyses to build predictive models using a five-fold cross-validation approach. Balanced accuracies greater than 70% within the training and test chemical sets were achieved for the models. Examination of the identified critical pathways revealed expected as well as novel contributions to the predictive models.

Phase II of ToxCast was launched this past year and will serve to broaden the chemical diversity and to provide data to evaluate the predictivity of the bioactivity signatures derived from Phase I data. It includes more than 800 additional chemicals in the same assays utilised in Phase I, plus new assays that cover additional technologies and biological pathways (Table 3). The Phase I chemical library consisted of primarily pesticidal active ingredients that had a wealth of available traditional toxicity data.

Table 2. Predictive bioactivity signatures developed from ToxCast Phase I results.

Endpoint	Brief Description of Bioactivity Signature	Reference
Reproductive function	Of the 309 chemicals in Phase I of ToxCast, 256 were linked to high-quality rat multigeneration reproductive toxicity studies in ToxRefDB. Reproductive toxicants were defined here as having achieved a reproductive lowest observed adverse effect level (rLOAEL) less than 500 mg/kg bw per day. 86 chemicals were identified as reproductive toxicants in rats; 68 of those with sufficient *in vitro* bioactivity to model. Each assay was assessed for univariate association with the identified reproductive toxicants. Significantly associated assays were linked to genes or sets of related genes and used for the subsequent predictive modelling. The primary genes identified were nuclear receptors, both steroidal and non-steroidal, and included the androgen receptor (AR), estrogen receptor alpha (ERα; ESR1), peroxisome proliferator-activated receptors, alpha (PPARα) and gamma (PPARα). A number of cytochrome P450 enzyme inhibition (CYP) assays, including aromatase (Cyp19a1), were also significantly associated with the reproductive toxicants. Interestingly, besides the human aromatase assay, rat CYP assays had increased association to the endpoint as compared to the human CYP assays. In addition to these genes and assay sets, individual assays representing cell-based markers of growth factor stimulation and cell signalling, including epidermal growth factor 1 (EGFR1), transforming growth factor beta 1 (TGF-ß1), vesicular monoamine transporter 2 (VMAT2), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were other positive indicators of reproductive toxicity potential.	Martin et al. (2011)

Endpoint	Brief Description of Bioactivity Signature	Reference
Prenatal development	Twelve features, representing single or groups of assays, were identified in building the model for rat developmental toxicity, of which eight were considered positive predictor: 1) RAR (retinoic acid receptor) feature included two transcriptional assays and one receptor-binding assay; (2) GPCR (G protein-coupled receptor) feature included the purinergic (PY2), opiate, opiate receptor-like 1 (ORL1), and the muscarinic cholinergic receptor 1 (M1) GPCR binding assays, which were amongst the highest correlating GPCRs; (3) TGFß (transforming growth factor beta) feature included two expression assays (transcript and protein); (4) MT (microtubule destabilisation) imaging assay feature; (5) SENS_CYP (cytochrome P450) feature included two CYP expression assays (CYP1A1, CYP2B6); (6) AP1 (activator protein 1) expression assay feature; (7) SLCO1B1 (solute carrier organic anion transporter family member 1B1) expression assay feature; (8) CYP feature enzyme assays (CYP2A2, CYP2B1) affected by a maximum of 27 chemicals. Four features were negative predictors: (1) HLA-DR (major histocompatibility complex) showing an increase in protein expression; (2) PXR (pregnane X receptor) expression assay; (3) IL8 (interleukin 8) feature included two assays showing decreases in protein expression; and (4) PGE2 (prostaglandin receptor) feature included decreases in protein expression. Seven features were identified in building the model for rabbit developmental toxicity, of which five were considered positive predictors: (1) CCL2 (chemokine ligand 2 or MCP1, monocyte chemotactic protein) feature included five small cytokine protein expression assays; (2) IL (interleukin) feature included protein expression assays of IL1a and IL8; (3) CYP feature included a CYP1A1 expression and CYP2A2 enzyme inhibition assay; (4) TGFß feature included two protein expression assays, (5) MESC (murine embryonic stem cell) feature included assays measuring a decrease in cell number and MHC (myosin heavy chain) expression. The negative predictors were (1) SULT2A1 (sulfotransferase) expression assay; and (2) PGE2 (prostaglandin receptor).	Sipes et al. (in press)

Endpoint	Brief Description of Bioactivity Signature	Reference
Vascular	A bioactivity signature for potential chemical disruption of blood vessel formation and remodeling was derived from the HTS data. Six assay targets emerged as most relevant to embryonic blood vessel formation based on biological plausibility and prevalence in the literature. In order of descending influence these were: down-regulation of the receptor tyrosine kinase (RTK) VEG-FR2; inhibition of the enzymatic activity of TIE2, an angiogenic RTK; down-regulation of the pro-angiogenic chemokine CCL2; perturbation of the Plasminogen Activating System (PAS) controlling extracellular ma-trix breakdown via up- or down-regulation of plasmi-nogen activator inhibitor type 1 (PAI-1/SERPINE1); up-regulation of the pro-inflammatory anti-angiogenic chemokine CXCL10; and perturbation of the PAS via up or down regulation of urokinase type plasminogen activator receptor (uPAR/PLAUR). Distinctly different correlative patterns were observed for chemicals with in vivo effects in rabbits versus rats, despite derivation of in vitro signatures based on human cells and cell-free biochemical targets, implying conservation but potentially differential contributions of developmental pathways among species. Follow-up analysis with anti-angiogenic thalidomide analogs and additional in vitro vascular targets showed in vitro activity consistent with the most active environmental chemicals tested here. These predictions implicate the embryonic vasculature as a target for environmental chemicals acting as puta-tive Vascular Disruptor Compounds (pVDCs) and illumi-nates potential species differences in sensitive vascular developmental pathways.	Kleinstreuer, et al. (in press)

The Phase II chemicals include additional food-use pesticides with a rich toxicological database, a number of drugs that failed during human clinical trials, representatives of several categories of high production volume (HPV) chemicals, and chemicals used as food additives which also have a rich toxicological database. However, the availability of traditional toxicity bioassays will be more limited than in Phase I, as we expect only about half of the chemicals will have a full complement of developmental toxicity, reproductive toxicity and chronic *in vivo* studies. Importantly however, the library

includes approximately 150 chemicals that were developed as pharmaceutical compounds but subsequently exhibited unexpected human toxicities. These chemicals, along with the accompanying human data, were donated by Pfizer, Merck, GSK, Sanofi-Aventis, Roche and Astellas. They will provide an important foundation on which to strengthen the models of predictive toxicity as they avoid the need to use animal models as surrogates for understanding potential human toxicity. The remaining chemicals in the Phase II library expand the diversity of chemicals and were selected to more adequately represent the types of chemical structures that would be the target of ToxCast should it be implemented to screen and prioritise chemicals for further testing. Similar to the varied availability of traditional animal toxicity data for the ToxCast chemicals, there is a wide variety of information on potential human exposures, with the pharmaceutical compounds having the most directly relevant data.

We are also conducting an extension of ToxCast—called the 'e1k' study—that will utilise the estrogen, androgen and thyroid related assays from the current ToxCast assay portfolio (approximately 80 assays in total) to screen an additional 1000 chemicals. This information will be used to help the EPA regulatory office in charge of the Endocrine Disruptor Screening Programme (EDSP) select chemicals for higher-level screening using the EDSP Tier 1 assay.   The ToxCast Phase I and II libraries and the e1k library are part of a larger US Government research collaboration called Tox21. Prior to the

NRC report in 2007, these efforts—which consisted of the National Toxicology Program, through its Roadmap (NTP, 2004), the NIH Chemical Genomics Center through its Molecular Libraries Initiative (*ncgc.nih/gov*), and the EPA through the ToxCast programme and Strategic Plan for Evaluating the Toxicity of Chemicals (US EPA, 2009)—were only loosely co-ordinated. Spurred on by the NRC report, those three government agencies entered into a Memorandum of Understanding in February 2008 (Collins et al., 2008) to bring their expertise and complementary capabilities to bear on transforming the conduct of toxicological evaluations. The US Food and Drug Administration joined the Tox21 consortium in 2010. Tox21 has formal working groups on chemical selection, assay selection, informatics and targeted testing (Kavlock et al., 2009). Initially the NTP and EPA each contributed approximately 1400 chemicals to an assay programme focused primarily on nuclear receptor and other cell signalling biology. This effort proved that quality data could be obtained, examples of which are starting to appear in the literature (Xia et al., 2009; Huang et al., 2011). Currently the consortium is completing construction of a 10,000 member chemical library, contributed equally by the three founding organisations. Screening will start in mid 2011, with a capacity of approximately 25-30 assays per year. The initial priorities will be on nuclear receptor-mediated pathways, and on stress-related pathways. Also in 2010, discussions began with the European Commission Joint Research Centre in Ispra, Italy to expand this to an international collaboration.

Table 3. Characteristics of chemicals included in HTS assays (see epa.gov/ncct/toxcast/ chemicals.html for complete listing of chemicals; note the e1k and Tox21 libraries are scheduled for public release in the autumn on 2011).

Compound Library	Characteristics	Toxicity Data	Human Exposure	Assays
ToxCast Phase I	Pesticidal actives	+++	++	~500
	Commercial chemicals	++	+/-	
ToxCast Phase II	Commercial chemicals	+/-	+/-	~700
	Pesticide actives	+/-	+	
	Failed pharmaceuticals	++	+++	
e1k	Commercial chemicals	+/-	+/-	~80
Tox21	Pesticidal actives			~50-100
	Commercial chemicals	+/-	+	
	All marketed	+/-	_/-	
	Pharmaceuticals	+/?	+++/?	

The large amounts of information originating from computational toxicology approaches such as ToxCast requires that data reduction and visualisation tools be developed to facilitate their interpretation and understanding. The ToxPi approach as proposed by Reif et al. (2010) is one such tool. In this approach, the experimental results are grouped into common domains of information. For example, if one had a number of assays that looked at various aspects of estrogen receptor biology (e.g., receptor-binding, co-activator recruitment, gene activation), these would be grouped together into a 'domain' for purposes of ranking. The rationale for domain grouping being that it is the weight-of-the-evidence for perturbations of estrogen receptor biology that is being sought, and not the results of any one assay that could yield false negative (or false positive results). The collective data within a domain are then normalised to the range [0,1] according to their relative potency, with chemicals having the higher potencies receiving a value closer to one. In the current implementation, which was developed for prioritising based on endocrine-related activity, domains are included for estrogen, androgen, thyroid and other nuclear receptors interactions all derived from *in vitro* bioassays; physico-chemical properties such as log P and total polar surface area derived from computational models; and disease pathway domains obtained from mapping the *in vitro* assays against several representations of biological pathways (e.g., KEGG and disease pathways). For each chemical, an index value called the ToxScore™ is calculated as a weighted combination of all data domains. Thus, the ToxScore index represents a formalised, rational integration of information from different domains. Visually, the ToxScore is represented as component slices of a unit circle, with each slice representing one domain (or related pieces) of

information. For each slice, distance from the origin (centre) is proportional to the normalised value (e.g., assay potency or predicted permeability) of the component data points comprising that slice, and the width (in radians) indicates the relative weight of that slice in the overall ToxScore calculation.

To date, much of the emphasis of the ToxCast programme has been to develop tools to prioritise chemicals for more extensive *in vivo* testing. Recently we have considered how the approach could be extended to provide more quantitative information on potential risk for data-poor chemicals. Important inputs to chemical risk assessments are estimates of the highest allowable exposure levels that are protective of human health. Typical acceptable exposure values such as the reference dose (RfD) are based on expensive and time consuming animal toxicity tests. Non-animal based methods to estimate safe exposure levels would be beneficial because there are tens of thousands of existing chemicals with little or no animal testing data, and hundreds more chemicals introduced into commerce every year. In Judson et al. (2011) we present an approach to derive screening-level estimates of allowable exposure levels for data-poor chemicals. The method uses knowledge derived from ToxCast data that informs how chemicals alter biological processes or pathways related to human disease and information from *in vitro* pharmacokinetic studies that provide input on metabolic clearance rates and protein binding. The former

allows extrapolation of activities recorded from in *in vitro* bioactivity assays to levels necessary to achieve similar steady-state blood levels (Rotroff et al., 2010) though a method we term reverse toxicokinetics. ToxCast and Tox21 HTS assays are combined with data on metabolism and pharmacokinetic modelling to estimate exposure levels reasonably expected to be without risk of chemically induced disease in human populations. The proposed HTRA approach is essentially a five-step process that calculates a 'biological pathway altering dose' (BPAD) useful in estimating acceptable exposure levels by: (1) identifying the biological pathways that are known to be the key connection between mode-of-action based risk assessment and HTS; (2) measuring the chemical activity in concentration-response to determine the concentration of a chemical that can perturb the biological pathway in cells (termed the 'biological pathway altering concentration' (BPAC)); (3) converting the HTS concentration-response to whole-animal dose-response using metabolic measurements and pharmacokinetic models to calculate the BPAD; (4) incorporating population variability and uncertainty; and (5) estimating the lower limit from the BPAD the (BPADL$_{99}$) below which there is minimal risk of the toxicity-related pathway being perturbed. As a proof-of-concept, BPADs were calculated for the estrogenic chemical, Bisphenol A, and for a group of conazole fungicides that exhibit toxicity via alterations in xenobiotic metabolizing enzymes. In both instances, close agreement with traditional risk assessment approaches were observed.

Conclusion

In summary, significant advances in the evaluation of HTS assays for predictive toxicology have been achieved over the past several years. Large, well-curated databases have been made available to the scientific community for independent analysis, and even larger databases are under construction. The next few years will witness an unmatched volume of information related to the biological targets and pathways by which environmental chemicals may cause chronic diseases and afford the opportunity to bring the vision of toxicology in the 21st century nearer to fruition.

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Embryonic Stem Cell Approaches Towards Biomarkers of Embryotoxicity

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Abstract

Many alternative methods for developmental toxicity testing have been developed over recent decades, but their implementation in regulatory toxicology has not shown the same pace. This paper contemplates various possible causes and discusses possible ways forward, taking the example of the embryonic stem cell test (EST). The addition of transcriptomics analysis to improve mechanistic effect assessment is reviewed, as are issues such as the definition of the applicability domain and the choice of test compounds for validation. The necessity to combine various tests with different endpoints into an integrated testing strategy is discussed. Also, analysis of existing databases of animal testing results together with human-derived data is put forward for the derivation of critical endpoints and mechanisms-of-action. This should promote the innovation of chemical hazard assessment towards a mechanistically-based approach, improving its scientific basis and accelerating the implementation of alternative methods.

Introduction

The development of alternative, animal-free tests for chemical and pharmaceutical safety has resulted in a wide variety of model systems that can be employed for assessing specific toxic effects of compounds. However, the implementation of alternative methods in regulatory frameworks has not shown the same level of success. This discrepancy may have different causes. First, the general idea that an *in vitro* test should replace an *in vivo* test 1:1 is not scientifically warranted in many areas of toxicity testing. Many different pathways of toxicity may occur simultaneously and interdependently, and this

complex situation cannot be mimicked sufficiently in a single *in vitro* test. This implies that a battery of alternative tests is needed, which should be combined in an integrated testing strategy, increasing the complexity of the hazard identification strategy.

Second, the now classical idea of validation, which was based on defining predictability by testing a series of chemicals with diverse structures and properties, has proven to be less informative than thought heretofore. Whereas for a limited set of up to 20 compounds a test and its prediction model may be defined towards a high level of predictability, the same test may not perform that well with a different set of compounds, as experience has shown. The notion emerges that alternative tests should be judged on the basis of being 'suitable' (REACH, 2006) rather than 'validated'. One important criterion for suitability could be the definition of the applicability domain of the test. This domain includes a description of the modes- and/or mechanisms-of-action represented in the assay. Knowledge of the applicability domains of assays allows a well-informed choice of combining complementary assays within the integrated testing strategy. For relatively complex assays such as the embryonic stem cell differentiation assay and the whole embryo culture for developmental toxicity testing, the definition of applicability domain is not straightforward. As a consequence, the predictive capacity of the test cannot be judged easily on the basis of testing diverse chemicals in the assay. This is much easier in a single mechanism test such as, e.g., an estrogen receptor-binding assay, which can be considered suitable as soon as the specificity of the assay, in this case receptor binding by estrogens only, has been proven with a limited set of chemicals.

Third, for complex assays such as those referred to above, validation could be done using classes of chemically-related compounds in order to assess the applicability of the assay for each class. This has been applied already decades ago by Kistler (1985) with retinoids in the limb bud micromass assay. He showed that retinoids could be tested in this assay with a good predictability of their relative *in vivo* developmental toxicity potential. This approach, however, assumes that the testing strategy should be applied case-by-case dependent on the type of compound tested.

Fourth, the regulatory arena is generally satisfied with the current hazard and risk assessment practice, in view of the absence of calamities, the relative simplicity of the system, and the wealth of historic information for comparison. A novel testing strategy on the basis of alternatives should therefore aim not only at reduction of animal testing but should provide added value as to scientific scrutiny regarding hazard identification. Novel targets that are currently not regularly assessed in hazard identification, such as developmental neurotoxicity and developmental immunotoxicity, for both of which clinical evidence of related early onset diseases is mounting, could

perhaps be tested efficiently in alternative assays and provide added value for hazard assessment (Tonk et al., 2010; Crofton et al., 2011). In addition, in-depth information about mode-of-action could be used to improve interspecies extrapolation, allowing informed assessment of relevance of findings for the human situation. This could reduce the number of false-positives that might include very useful compounds that are now dismissed on the basis of irrelevant information. The challenge for the scientific community is to involve the regulatory world in the development and implementation of alternatives. This requires extensive communication—ideally throughout the process of development, characterisation, standardisation, and validation of novel assays. In addition, the definition of the testing strategy should be an integral part of this process.

Fifth, although replacement of animal testing may be the ultimate goal for many workers in the field of alternatives, it is worthwhile to consider a stepwise approach, in which the first aim could be the definition of an *in vitro* test battery that could be used as a filter predetermining which compounds should enter animal testing as a last resort. This should increase efficiency of safety testing and ensure that only those compounds enter into animal testing that are most promising for their foreseen application. Such an approach will be more easily acceptable from a regulatory perspective and will allow the building of a testing strategy on the basis of the most important endpoints of toxicity as determined from classical animal testing. Thus, the way forward towards

integrated testing strategies, built on the backbone of alternative assays, could be threefold, and consist of: 1) a database analysis of existing expertise to identify key endpoints of toxicity; 2) development of alternative assays for key endpoints of toxicity; and 3) combining alternative assays in an integrated testing strategy (van der Burg et al., 2011). By building on existing expertise, innovations could be more easily acceptable in the regulatory framework. Moreover, additional expertise could come from human non-testing data, informing about toxicity pathways, such as exemplified in the US National Research Council vision of 'toxicity testing in the 21st century' (NRC, 2007).

Embryonic Stem Cell Test (EST)

The EST was developed by Heuer et al. (1993) and was based on the interference of chemicals with the differentiation of embryonic stem cells into beating cardiomyocyte foci in culture. The test was validated in the largest interlaboratory ECVAM validation study performed at the time (Genschow et al., 2002). This validation study has been instrumental in feeding subsequent discussion about the concept of validation. Twenty compounds were tested in four independent laboratories, giving rise to close to 80% predictability. The assay was subsequently launched on the ECVAM website as an officially validated assay, ready for regulatory implementation. However, follow-up testing with additional compounds by several laboratories did not give rise to favourable results (Paquette et al., 2008). This was partly attributed

to the prediction model used, which was purely mathematically derived by biostatisticians, the biological relevance of which was unclear (Marx-Stoelting et al., 2009). In addition, the applicability domain of the assay, considered as an assay for 'developmental toxicants', was possibly estimated too widely. It was also suggested that additional endpoint parameters such as differential gene expression assessment could improve the predictability of the assay.

We have embarked on transcriptomics readout in EST with the aims to enhance predictability, increase mechanistic information, and define applicability domain in more detail. Each of these aspects could contribute to facilitation of regulatory implementation along the various lines indicated in the Introduction above. Transcriptomics proved useful in describing compound effects in EST in a series of experiments in our laboratory (van Dartel & Piersma, 2011). A gene set was derived which gave rise to over 80% predictability in the test, on the basis of around 20 compounds (Pennings et al., 2011). The question remains, however, as to what steps can be practically taken to accelerate regulatory acceptance of such an assay, if warranted. In the following, several aspects are highlighted which we believe could help improve knowledge on the performance of the assay and its possible place within an integrated testing strategy, and help increase confidence in the system in order ultimately to promote regulatory acceptance.

Ways Forward to Accelerate Implementation of EST

Additional Compounds

'The proof of the pudding is in the eating'. More compounds need to be tested in order to enhance the database of assay results, and with that the predictive gene set and the confidence in the assay. We have already performed additional studies to improve the predictive gene set, but more work is definitely needed (Pennings et al., 2011). The choice of compounds is always limited by available information about *in vivo* developmental toxicity. Various attempts in the past have not given a panacea for a standardised validation compound group (Brown, 2002). A recent initiative recognises that a simple positive-negative classification does not suffice in the light of Paracelsus' statement that "the dose makes the poison". It proposes to define 'exposures' rather than compounds as positive or negative (Daston et al., 2010). Exposures are defined in this context as a compound in combination with a certain target organ dose, mimicked *in vitro* by a culture medium concentration. In addition, rather than for diverse groups of chemicals, validation may be performed for structural classes of chemicals as discussed above, which informs about the applicability of the test for the class of compounds assayed.

Gene Expression & Protein Functionality

In order to establish the functionality of gene expression changes, we have

compared gene and protein expression changes in the system (Osman et al., 2010). We observed a series of gene-protein pairs that were regulated in EST, showing that early gene expression changes in the cases identified resulted in functional consequences in the form of protein synthesis. This finding confirmed the functional nature of the transcriptomic approach and indicates that the assay does not just measure artefacts, enhancing the confidence in the general approach.

In Vitro – In Vivo Potency Ranking

We performed several studies to establish potency ranking of chemicals within a structural class and compare this with the *in vivo* situation (de Jong et al., 2011). These studies showed remarkable coherence between *in vitro* and *in vivo* rankings, which could be improved by incorporating kinetic modelling of target exposure *in vivo*. These aspects help in gaining confidence in the significance of *in vitro* critical exposure levels in view of *in vivo* potency of compounds.

Threshold of Adversity In Vitro

An as yet unresolved issue is how to define the threshold of adversity from an *in vitro* dose-response. Measures of cytotoxicity may or may not be artefactual *in vitro*, and in a differentiating system one would like therefore to discriminate differentiation inhibition from effects on cell viability. Our transcriptomics approach allowed us to discriminate through the expression changes of gene sets related to various functional aspects of the cells in culture. For example, a dose-response of flusilazole showed that developmental related gene sets changed expression at lower doses than viability related gene sets (van Dartel et al., 2011). In addition, the sterol biosynthesis gene set, representing the mechanism of antifungal action of the compound, showed a unique dose-response. These findings support the added value of transcriptomics to discriminate among various mechanisms of compound action in EST, again increasing knowledge of the biology of the system, its relevance to *in vivo* action, and enhancing confidence in the soundness of the assay.

Applicability Domain: In Vitro – In Vivo Gene Expression Comparison

We did additional comparisons of compound-induced transcriptomics responses in *in vitro* and *in vivo* experimental systems, in order to determine whether the gene expression inductions found *in vivo* would mimic those *in vitro* (Robinson et al., 2011). These analyses clearly showed commonalities among developmental systems as opposed to non-developmental models in terms of the regulation of developmental gene sets, confirming the specificity of the response and the commonalities between *in vitro* and *in vivo* developmental models.

Alternative Differentiation Routes: Neural Differentiation

We considered that the cardiac EST, even with additional transcriptomics readout, does not provide a panacea for

detecting all developmental toxicants. For example, methylmercury gives rise to misclassifications in the cardiac EST. Following up on existing initiatives (Stumann et al, 2009), we therefore also embarked on the design of a neural EST, incorporating differential gene expression as an additional readout system (Theunissen et al., 2011). This approach clearly showed methylmercury to affect neurodevelopment, in a way that mimicked the known mode-of-action of the compound *in vivo*. Further development of this model and comparison with the cardiac EST will inform about the added value of combining both tests in a battery approach. Other differentiation routes such as towards bone may also be of added value.

Human ES: Remove Interspecies Extrapolation

The default use of animal cells in alternative assays for the prediction of human hazard has probably primarily been driven by availability and technical convenience. However, in the interest of removing the necessity for interspecies extrapolation, the use of human cells for human hazard assessment would be a principal advantage. The disadvantage of the more laborious and relatively complex culture conditions currently available for human embryonic stem cell lines could be overcome by investing in additional research towards more simplistic culture techniques and assay conditions (Stumann et al., 2009). This development is in line the vision of the US National Research Council report on "Toxicity Testing in the 21st Century", which advocates the use of

human based assays wherever possible (NRC, 2007).

Embed in Hazard Assessment Testing Strategy

In addition to investing in optimising and characterising individual alternative assays, the consideration of their use in an alternative testing strategy is of equal importance. It is clear that in order to cover the full complexity of developmental toxicity it will be necessary to combine in the strategy assays that cover complementary endpoints and mechanisms. The ReProTect project has performed a first study in that direction, the ChemScreen project is one of the arenas where such a strategy is currently being designed (Schenk et al., 2010, van der Burg et al., 2011).

Promote & Facilitate Acceptance in International Regulatory Frameworks

Considerable efforts will be needed to convincingly promote alternative testing strategies as replacements, if even partial, of the current animal-based testing paradigm. This can only be successful if it can be shown that the alternative approach not only at least guarantees current levels of chemical safety, but moreover has added value for hazard assessment beyond the reduction of animal use. Especially demonstrating a higher informative value as to human hazard prediction on the basis of mechanistic knowledge would be important, as it should reduce false negative and false positive findings inherent in current animal testing. Reductions in

cost and time, allowing more chemicals to be tested in, e.g., the REACH programme, provide additional argumentation.

Conclusion

The development and validation of alternative assays for developmental toxicity testing has received increased interest in view of the need to reduce animal use and the necessity to improve risk assessment of chemicals through mechanistically informed hazard assessment. Innovative approaches such as transcriptomics allow detailed assessment of *in vivo* as well as *in vitro* effects at the molecular level. These approaches facilitate both a more detailed description of the effects of chemicals as well as improve *in vitro* to *in vivo* extrapolations. Embedded in an integrated testing strategy, a combination of alternative methods covering essential endpoints and pathways of toxicity is expected to provide improved hazard assessment of chemicals. The enhanced mechanistic basis of hazard assessment, resulting in fewer false positives and negatives, in combination with reduced animal use and testing time and cost should synergise towards accelerated implementation of alternative methods in chemical safety assessment.

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The Virtual Embryo

A Computational Framework for Developmental Toxicity

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Overview

EPA's 'Virtual Embryo Project' (v-Embryo™) is focused on the predictive toxicology of children's health and developmental defects following prenatal exposure to environmental chemicals. The research is motivated by scientific principles in systems biology as a framework for the generation, assessment and evaluation of data, tools and approaches in computational toxicology. The long-term objectives are to: determine the specificity and sensitivity of biological pathways relevant to human developmental health and disease; predict and understand key events during embryogenesis leading to adverse fetal outcomes; and assess the impacts of prenatal exposure to chemicals at various stages of development and scales of biological organisation.

Background

21st Century tools are now providing high-dimensional data at the molecular and cellular level that can advance predictive toxicology

Complex biological systems such as a developing embryo have traditionally been studied by reductionism, whereby the system is divided into increasingly simpler parts and knowledge is integrated at increasingly more fundamental levels of biological organisation. The concepts, principles and possible solutions gained by this approach are evident in large-scale initiatives such as the human genome project and the information explosion in biomedical knowledge. Newer technologies for rapid and automated screening of large molecular libraries for broad biological responses have led to a new vision and strategy of toxicity testing for the 21st Century [1]: move away from animal testing toward high-throughput assays to understand how chemicals perturb cellular

functions, establish relationships between *in vitro* perturbation (toxicity pathways) and *in vivo* outcomes (adverse outcome pathways), and provide broader coverage of chemicals and biological activities with less dependence on animals (3Rs).

Predictive models built from ToxCast Phase-I (309 chemicals) include apical endpoints in ToxRefDB [2-4] and pathways from biomedical literature [5-7]. The general idea has been to mine signatures of toxicity from *in vitro* high-throughput screening (HTS) data and prioritise chemicals by specific endpoints and pathway targets. Early results for developmental toxicity [4] suggest that
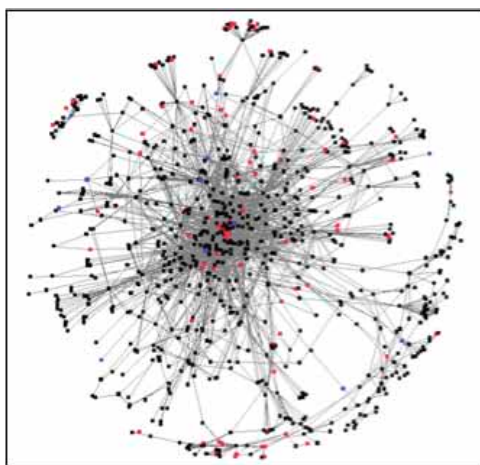

Figure 1. Chemical-target perturbation network for developmental toxicity. Predictive model built from AC50s for 309 Phase-I chemicals tested in 662 in vitro assays and mined against the ToxRefDB for 17 developmental endpoints in pregnant rats (251 chemicals) and rabbits (234 chemicals) [4]. Nodes annotated by GO biological process and edges represent linkages; black nodes: significant univariate features; blue nodes: angiogenesis; red nodes: multivariate features selected by a simple linear model.

susceptible processes form a complex web of interactions (Figure 1). This may account for species differences, target tissue and stage susceptibility. As such, having scientifically accepted predictive tools will enable the more efficient and rational use of animals as testing is directed to the highest priority chemicals and pathways.

Computational models are needed to analyse complex systems and gauge the biological plausibility of predictive signatures

Cellular and molecular information captured by *in vitro* profiling may be more closely linked to critical pathways than could be inferred from *in vivo* exposure in pregnant rats or rabbits; however, reconstructing toxicity pathways from these data remains an important challenge for the HTS paradigm. The number of parts that compose an embryonic system is massive; each part in itself has its own developmental trajectory, is subjected to weak physical relations depending on network topology, and may be influenced by overall network dynamics during perturbation. Resiliency of a complex adaptive system to perturbation increases as parts are assembled into higher levels of organisation. As such, the ways in which parts come together must be looked at very closely during normal and abnormal development.

Modelling and simulation tools are necessary to understand and analyse the complex relationships observed between signaling networks and multicellular behaviours. The impact of

chemical disruption on core functions such as molecular clocks, spatial gradients, molecular machines, growth and differentiation cannot be easily comprehended without computational (*in silico*) models of embryogenesis and relevant quantitative data. Virtual models thus capture the flow of molecular information across biological networks and process this information into higher-order responses. Responses to perturbation are dependent on network topology, system state dynamics, and collective cellular behaviour.

The HTS paradigm together with computational systems biology forms a powerful tandem for understanding complex adaptive systems

EPA's Virtual Embryo project (v-Embryo™) is building computer-based virtual models of tissues and physiological processes that are network-based, multi-component, and distributed across several scales of biological and physical complexity [8]. The goal is to deploy virtual models for toxicological assessment of developmental processes, recapitulating a morphogenetic series of events driven by a myriad of molecular targets, biochemical pathways, and signalling networks.

The basic idea of a Virtual Embryo is to create an array of systems models that represent key aspects of embryonic development. For example, blood vessel development, early limb development, and retinal morphogenesis are some examples of embryonic systems that are driven by cell signalling pathways and complex

cellular behaviours. Building capacity to reconstruct key events in toxicity requires computational models whereby each biological cell autonomously integrates cues from its direct environment and acts according to its own blueprint. The co-ordinated activities of many thousands of cells in a system can thus be simulated, leading to higher-order emergent properties that represent a systems-level biological output.

Virtual Embryo deploys cells as 'agents'. This type of model is referred to as cell-agent-based because the individual cell is the smallest unit capable of an autonomous decision. A cell-agent-based model is ideal for modeling development because cells are the biological unit of the embryo and the computational unit of a virtual embryo. This type of model is also referred to as multi-scale because it integrates information across different biological scales: molecular information such as internal clocks, biochemical gradients, and gene regulatory networks; cellular properties such as growth, adhesion, and differentiation; and tissue-level properties such as homeostasis, morphogenesis, and repair. Functionality depends on information about individual cellular behaviour(s) and knowledge of the system coded into the software. From multi-cellular interactions emerges a morphogenetic series of events.

Cell-agent-based models in Virtual Embryo are executed from a computer programme coding specific cellular behaviours using the Python programming language. Specific rules for molecular pathways and

cellular behaviours are captured from the vast scientific literature in a virtual tissue knowledgebase (VT-KB). The software to implement multicellular interactions is CC3D open access tissue simulation environment (*compucell3d.org*) and can simulate morphogenesis as individual cells grow, divide, differentiate, adhere, migrate, and die based on systematic rules and local signals. Anatomical features of the developing embryo emerge as the simulation unfolds and can be compared at different stages to the real system to validate model performance.

Application of virtual models in predictive toxicology

Cell-agent based models are useful for modelling developmental toxicity by virtue of their ability to accept data on many linked components and implement a morphogenetic series of events. These data may be simulated (e.g., what is the effect of localised cell death on the system?) or data derived from *in vitro* studies (e.g., ToxCast HTS data). In the latter case perturbed parameters are introduced as simple lesions or combinations of lesions identified from the data, where the assay features have been annotated and mapped to a pathway or cellular process implemented in the virtual model. Whereas ToxCast predictive models are built with computer-assisted mapping of chemical-assay data to chemical-endpoint effects, the virtual tissue models incorporate biological structure and thus extend the HTS data to a higher level of biological organisation. As such, a developing system can be modelled and

perturbed 'virtually' with toxicological data and then the predictions on growth and development can be mapped against real experimental findings.

Blood vessel development has been selected as a case study. In the embryo, endothelial cells assemble into a primary capillary network of capillaries (vasculogenesis) and new capillaries sprout from pre-existing vasculature (angiogenesis). This was modelled in CC3D for several dozen parameters derived from the scientific literature, and tailored to accept data derived from ToxCast. Importantly, a number of ToxCast assays play key roles in the major angiogenic pathways, including VEGF signaling, chemokine signalling and plasminogen activator system. A predictive model was built for the Phase I chemical library, leading to a prioritisation of 309 chemicals based on the predicted potential for vascular disruption [7]. Among the strongest putative vascular disruptor compounds (pVDCs) we observed quite different signatures across the major angiogenic pathways.

Given that imbalances in angiogenesis can promote (tumourigenesis) or suppress (teratogenesis) tissue growth, we modelled different ToxPi signatures with CC3D to assess the potential for vascular disruption in a virtual model of angiogenesis. Thalidomide is an example that causes foreshortening of the arms and legs through an anti-angiogenic mode-of-action. We explored this predicted relationship by setting up a multicellular simulation of angiogenesis. Running the simulation with lesions from

ToxCast assays revealed the potential consequences to angiogenesis, based on the scientific knowledge, empirical data, and emergent systems-level properties (Kleinstreuer, work in progress).

Challenges & Next Steps

Virtual Embryo models can be used in several ways to extrapolate predictions from cell-level data to developing organ systems, although a good amount of biological detail is needed to build cell-agent-based models and asses model performance. This exploits the advantages of a screening-level approach such as *in vitro* profiling, in which HTS data and *in vitro* assays probe lower levels of biological organisation that are faster and less expensive than traditional animal studies to analyse key events in a potential adverse outcome pathway. Whereas computational modelling can significantly aid generating *in vivo* hypotheses from the *in vitro* data and yield insight into systems-level behaviour, the virtual models must be grounded in results from actual experimentation.

A toolbox of virtual tissue models that recapitulate critical embryonic processes provide biological context to HTS data to predict, from a computer simulation, the consequences at a higher level of biological organisation. As the models are tested and refined we anticipate users can exercise complex scenarios across a broad range of parameter sweeps to prospectively (predictive models) or retrospectively (analytical models) assess the nonlinear behavior of a complex adaptive system.

Disclaimer
The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the US Environmental Protection Agency. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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The GARD Test

A Novel Assay for Prediction of Skin Sensitisers

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Background & Objectives

Prediction of sensitising properties of chemicals used within industry and research is an important aspect of safety assessment of chemicals. Such predictions are currently performed with the use of the mouse Local Lymph Node Assay (LLNA) (Basketter et al., 2002). Due to European legislation that regulates the use of animals within, e.g., cosmetic and pharmaceutical industries, the need of novel assays is urgent. Yet to date, no validated non-animal replacements are available for identification of sensitising substances. An *in vitro* alternative to these animal models should exhibit improved reliability, accuracy and importantly correlate to human reactivity. The objective of the here reported research has thus been to develop *in vitro* assays for prediction of sensitising chemicals.

GARD—the 'Genomic Allergen Rapid Detection' test (Johansson et al., 2011)—is such an assay, which has been developed for assessing the ability of a compound to induce skin sensitisation, which results in the disease known as allergic contact dermatitis (ACD). GARD is based on MUTZ-3 (Masttersson et al., 2002), a myeloid cell line with a phenotype and transcriptional profile similar to dendritic cells (DCs), which are essential for the initiation of the immune response leading up to ACD. The readout of the assay is the analysis of the transcriptional levels of 200 genes, which has been identified as differentially regulated in sensitisers, compared to non-sensitisers. Following data acquisition from DNA microarrays, the predictions are performed with a machine-

learning algorithm called Support Vector Machine (SVM) (Cortes & Vapnik, 1995).

In this report, we summarise the development of the GARD test. We will discuss the finding of the predictive genes, their function and relevance in the immunologic mechanisms leading to skin sensitisation, estimate the predictive power of the assay and relate it all to the deliverables and scientific goals that were set up in 2010.

Deliverables

Identify Predictive Genomic Signatures for MUTZ-3 Progenitors Related to Key Sensitisers

The GARD assay was developed using 40 reference chemicals, including 20 sensitisers and 20 non-sensitisers (Table 1). The toxic effect of these chemicals on MUTZ-3 was assessed using a standardised Propidium Iodide protocol. In order to measure immunological

effects, rather than effects induced by necrosis, all future stimulations were done in the concentrations yielding 90% relative viability (rv90).

For the microarray experiments, MUTZ-3 was seeded in wells at a cell concentration of 200,000 cells/ml. Stocks of each of the reference chemicals were added, so that the final in-well concentration corresponded to the rv90 values. Following an incubation of 24h, RNA was isolated and cDNA prepared. Hybridisation, washing and scanning of Affymetrix Human Gene 1.0 ST Arrays were performed according to standardised protocols. All stimulations were performed in triplicates and vehicle controls were included. After quality control of the arrays, the data set ready for analysis consisted of 137 arrays, each with data from measurements of 29,141 transcripts. This entire procedure, which is also the suggested protocol for the assay, is summarised in Figure 1.

Statistical analysis of the high dimensional
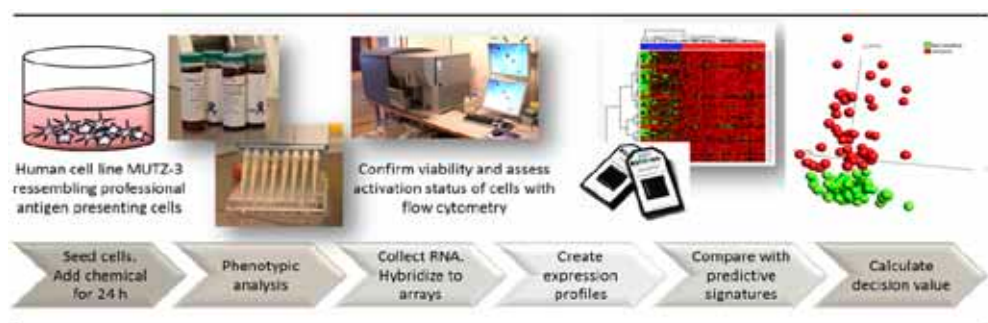

Figure 1. The GARD test principle. The MUTZ-3 cell line are seeded in wells, and incubated with test substances for 24h. RNA is isolated and hybridised to arrays. Following data acquisition, the transcription levels of the predictive signature (200 genes) are analyzed, and each sample is given a decision value as either sensitiser or non-sensitiser, based on SVM classifications.

Table 1. List of reference chemicals and their respective vehicle and concentration used in assay development. 1) Kathon CG is a mixture of MC/MCI. The concentration of this mixture is given in %.

Compound	Potency	Vehicle	Concentration in Culture (µM)
Sensitisers			
2,4-Dinitrochlorobenzene	Extreme	DMSO	4
Oxazolone	Extreme	DMSO	250
Potassium dichromate	Extreme	Water	1.5
Kathon CG (MC/MCI)	Extreme	Water	0.0035%1
Formaldehyde	Strong	Water	80
2-Aminophenol	Strong	DMSO	100
2-nitro-1,4-Phenylendiamine	Strong	DMSO	300
p-Phenylendiamine	Strong	DMSO	75
Hexylcinnamic aldehyde	Moderate	DMSO	32.24
2-Hydroxyethyl acrylate	Moderate	Water	100
2-Mercaptobenzothiazole	Moderate	DMSO	250
Glyoxal	Moderate	Water	300
Cinnamaldehyde	Moderate	Water	120
Isoeugenol	Moderate	DMSO	300
Ethylendiamine	Moderate	Water	500
Resorcinol	Moderate	Water	500
Cinnamic alcohol	Weak	DMSO	500
Eugenol	Weak	DMSO	300
Penicillin G	Weak	Water	500
Geraniol	Weak	DMSO	500
Non-sensitisers			
1-Butanol		DMSO	500
4-Aminobenzoic acid		DMSO	500
Benzaldehyde		DMSO	250
Chlorobenzene		DMSO	98
Diethyl phthalate		DMSO	50
Dimethyl formamide		Water	500
Ethyl vanillin		DMSO	500
Glycerol		Water	500
Isopropanol		Water	500
Lactic acid		Water	500
Methyl salicylate		DMSO	500

Compound	Potency	Vehicle	Concentration in Culture (µM)
Octanoic acid		DMSO	500
Propylene glycol		Water	500
Phenol		Water	500
p-Hydroxybenzoic acid		DMSO	250
Potassium permanganate		Water	38
Salicylic acid		DMSO	500
Sodium dodecyl sulphate		Water	200
Tween 80		DMSO	500
Zinc sulphate		Water	126

data, using a t-test comparing the groups sensitisers and non-sensitisers, with false discovery rate as a means to correct for multiple hypothesis testing, thousands of significant genes were found to be differentially regulated in cells stimulated with sensitisers, as compared to cells stimulated with non-sensitisers and vehicle controls. For further feature selection, we utilised an algorithm for backward elimination. This feature selection algorithm identified the 200 genes that collectively provide the best predictive power. The discriminatory properties of these genes are illustrated in Figure 2.

Confirm the Validity of Identified Markers Involved in Sensitisation in an Extended Test Set of Sensitisers

In order to validate the prediction signature and estimate the performance of the assay, 30% of the data was randomly removed, called a test set. With the remaining 70%, called a train set, the entire procedure of identifying the top 200 predictors was

repeated. A machine learning method called Support Vector Machine (SVM) was trained with these 200 predictors using the train set, and the model acquired was then used to predict the samples in the test set as either sensitisers or
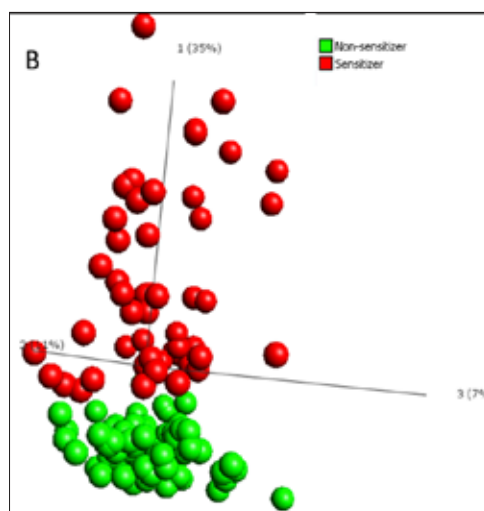

Figure 2. Principal component analysis of data: Complete separation between sensitisers (red) and non-sensitisers (green) is observed with PCA, when studying the 137 samples and the 200 genes of the 'prediction signature'.

non-sensitisers. This entire process was repeated 20 times, each time with a new random division into train and test set. The results from these experiments are showed in Figure 3. The performances of the predictions were evaluated using the 'receiver operating characteristic' (ROC) curve (Lasko et al., 2005). The average area under the ROC curve was 0.98, which indicates near perfect classifications, with an estimated accuracy of 99%. While this method does not validate the 'prediction signature' as such, since a slightly different signature is achieved in each iteration, it does indeed validate the method by which the prediction signature was identified. With no external test set at hand, it should be emphasised that this is an estimated accuracy that needs to be validated with additional reference chemicals, as discussed below in the Next Steps section below.

Furthermore, the validity and immunological relevance of the identified markers were examined using 'ingenuity pathway analysis'. The 200 genes were fed into the software, allowing it to investigate what canonical pathways these regulated genes are involved in. The most densely populated pathways were, in descending order, NRF2-mediated oxidative response, xenobiotic metabolism signalling, protein ubiquitination pathway, LPS/IL-1
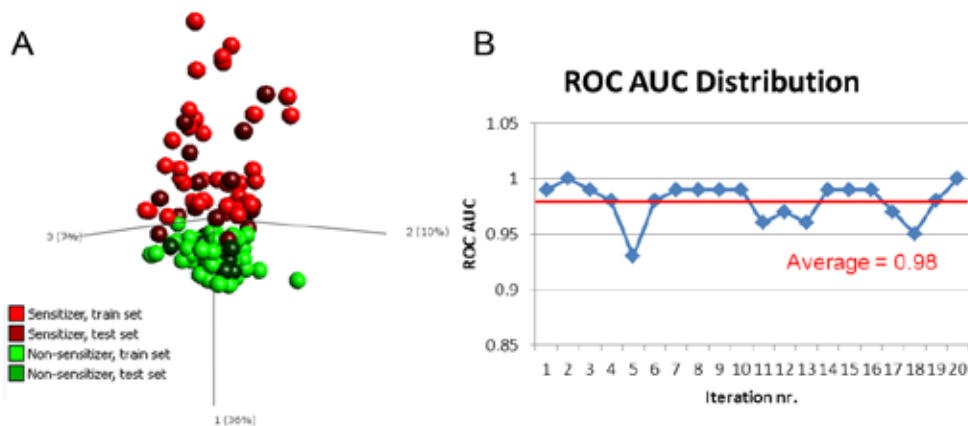
Figure 3. Validation of the selection procedure of the 'prediction signature': The method by which the 'prediction signature' was constructed was validated by repeating the process on 70% randomly selected data (training set). The remaining 30% of data was used as a test set for signature validation. The process was repeated for 20 iterations. A) A representative PCA of one of the 20 iterations, which demonstrates that the 'test gene signature' can separate skin sensitisers from non-sensitisers. Only the samples of the 70% training set, displayed in bright colours, were used to build the space of the first three principal components. The test set samples, displayed in dark colours, were plotted into this space based on expression levels of the analytes in the 'test gene signature'. B) An SVM was trained on the 70% training set, and validated with the 30% test set. The areas under the ROC curve from 20 such randomisations are plotted, yielding an average AUC value of 0.98.

mediated inhibition of RXR function, aryl hydrocarbon receptor signaling, protein kinase A signaling and TLR signalling. Interestingly, all of these pathways are induced by foreign low-molecular-weight compounds, called xenobiotics, the effects of which are precisely what we want to measure. In addition, a number of these pathways are involved in innate immune response signaling, such as the NRF2-pathway, the aryl hydrocarbon receptor signaling and TLR signaling, all of which leads to transcription of genes mediating cytoprotective effects and DC maturation, which would further support their validity as sensors for compounds leading to skin sensitisation.

Next Steps

In this section, we present four defined projects that summarize the next steps for further development and validation of the GARD assay.

Project 1: Validation of the Prediction Signature & Performance Evaluation of the Assay

The performance of the assay has been estimated with cross-validation, in an unbiased and statistically correct manner. However, even though the number of chemicals tested is relatively high, an additional data set with blinded compounds is essential to validate whether the assay truly performs as estimated by the random subdivisions into training and test sets. At present, a panel of additional chemicals

has been identified, and laboratory work and data analysis will be performed during the second half of 2011.

Project 2: Analysis of Pathway Regulation Induced by Individual Chemicals

The genes of the prediction signature are involved in a number of signalling pathways, which are all associated with recognition of foreign substances, innate immune responses and cellular stress. These pathways were identified as being the most densely populated pathways by the 200 transcripts in the prediction signature. As discussed, these 200 transcripts were found by looking at all sensitizers as a group, compared to non-sensitizers. However, it would be of great interest to see what pathways are regulated by the individual compounds in the reference chemical panel. Such an analysis could provide information that could be used for primarily two causes. Firstly, sensitisers could be further grouped and subdivided by their mechanisms of action, which in turn could be correlated to physical properties of the compound, such as peptide reactivity and potency as determined by the LLNA. Secondly, such information could provide great insight into the mechanism of skin sensitisation. The work is currently ongoing, and is hoped to be finalised in late 2011.

Project 3: Investigation of Assay Transferability to Different Technical Platforms

While GARD has been developed using DNA microarray technology, the assay might benefit from a transfer to a different technical platform, such as RT-PCR. In the end, the final platform for the assay depends on how user-friendly the assay is, what level of expertise is required of the operators, and the cost of material and reagents. A transfer to a different platform might require a reduction of the prediction signature size. Predictions have indeed been performed using as few as 11 genes, with positive results (data not shown). However, the prediction signature remains at 200 genes at present, to avoid biasing the model to the data set at hand. Instead, an additional set of reference chemicals, which will be used for validation of the prediction signature, as discussed above, might also serve as a means to reduce the signature size, by investigating whether all 200 genes are required for correct predictions. Investigation of the applicability of RT-PCR in the GARD assay is planned to start in early 2012.

Project 4: Investigation of GARD's Predictive Power for Respiratory Sensitisers

Low-molecular-weight compounds can not only cause ACD, but they can also sensitise the human respiratory tract, leading to syndromes similar to those caused by inhaled protein allergens, such as asthma and rhinitis. While this is a health concern of equal priority as skin sensitisation, less progress have been made with the development of *in vitro* assays for prediction of respiratory sensitisers. To date, only skin sensitisers have been investigated using the GARD setup. However, a panel of respiratory sensitisers has been identified, that will be used in the GARD setup. The goal is to see whether the current setup of MUTZ-3 and the prediction signature is able to predict also respiratory sensitisers, or if another set of biomarkers can be identified as predictive for this group of chemicals. The laboratory work within this project is complete, and data analysis will be performed during the second half of 2011.

Publications

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Skin Sensitisation
Modelling the Human Adverse Response

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Background & Objectives

In 2004, Unilever initiated a long-term research and capability development programme designed to evaluate and implement a new conceptual approach for assuring consumer safety without animal testing[1,2]. Unilever's approach for safety assessment is risk-based, meaning that all available data on a new ingredient (including predicted levels of consumer exposure during product use) are used to assess the level of risk posed by its proposed consumer use. The scientific challenge we are investigating is how, in the future, novel *in vitro* data and *in silico* predictions may be used within this risk-based framework. Within our Skin Allergy programme we have made a long-term investment to define and explore the key toxicity pathways that drive the disease process (i.e., allergic contact dermatitis) in humans, as we believe this mechanistic understanding will form a sound basis for our non-animal risk assessment approach. Here we share our progress to date as well as highlighting where we believe the key challenges for the future exist[3].

Current Risk Assessment Approach for Skin Sensitisation

Decisions about the safety of consumer products (such as soaps, body lotions and toothpaste) are made on the basis of a risk assessment (RA), in which data on the potential hazards of the ingredients are interpreted in the context of the likely exposure to the product. Allergic contact dermatitis (ACD) is an eczematous skin reaction, which results from a specific, delayed type hypersensitivity response to a small molecule (chemical) allergen[4]. To ensure that ingredients in our products do not induce skin sensitisation (and hence do not cause ACD in consumers), our current RA approach uses information on the concentration of the ingredient in the product, and on how the product is used by consumers, together with hazard characterisation data, to assess the risk to humans and thereby inform the safety decision[5,6].

When considering consumer exposure, it is recognised that not all consumers will use products in the same ways with respect to: how much they use them, how frequently they use them, and even the mechanics of how they use them (e.g., how long a rinse-off product is left on). This uncertainty has to be addressed within the RA. The way that a product will be delivered will also vary, depending on the applicator (novel or otherwise) and the product concept. Exposure information for various product types has been generated to inform RAs, e.g., those which have been commissioned by industry groups such as the European Cosmetics, Toiletries and Perfumery Association (COLIPA[7-9] and the US Personal Care Products Council (PCPC[10]). Information on the predicted exposure scenario for the consumer product and the existence of historical data on the same or chemically-similar ingredients is then used to guide the generation and interpretation of hazard characterisation data.

If we consider the generation of hazard characterisation data relating to skin sensitisation, two broad categories exist today; structure-activity evaluations, e.g., (quantitative) structure-activity relationships (Q)SARs, and the predictive animal tests, e.g., the mouse Local Lymph Node Assay (LLNA)[11]. The mouse LLNA is an accepted animal test method for characterising skin sensitisation potential[12], which is performed to determine a stimulation index (the extent of chemical-induced cell proliferation observed in the lymph nodes, relative to a vehicle control) for a range of chemical doses. For sensitisers, these data can be used to establish a dose-response relationship and assign the chemical to a potency class, from 'weak' to 'extreme'[13-14]. Once generated, LLNA data can be used to predict a 'no observed adverse effect level' (NOAEL) in humans[15], which can the be used to derive a theoretical safe level of exposure to the material using a quantitative risk assessment (QRA) approach[5,6]. The outcome of the QRA can also be interpreted along with any existing information, such as history of use or clinical data relating to the material in question (or similar benchmark materials), in order to reach an overall risk-based safety decision.

Non-Animal Risk Assessment Approach for Skin Sensitisation

The immunobiology of skin sensitisation has been investigated for many years and, as a consequence, is relatively well understood[15-19] (Figure 1). Our current mechanistic understanding is that in order to induce a sensitiser-specific T cell response and ACD, a skin sensitiser must be able to penetrate the skin, bind covalently to skin proteins (termed haptenation), and induce sufficient skin inflammation to activate skin-resident dendritic cells (DCs), thereby ensuring that the haptenated proteins are uptaken by activated DCs and transported to the draining lymph nodes (dLN) where they are processed and presented to hapten-specific T-cells. T-cell activation is dependent upon the successful binding of the T-cell receptor (TCR) to a complementary hapten/peptide-MHC complex displayed on the DCs, followed
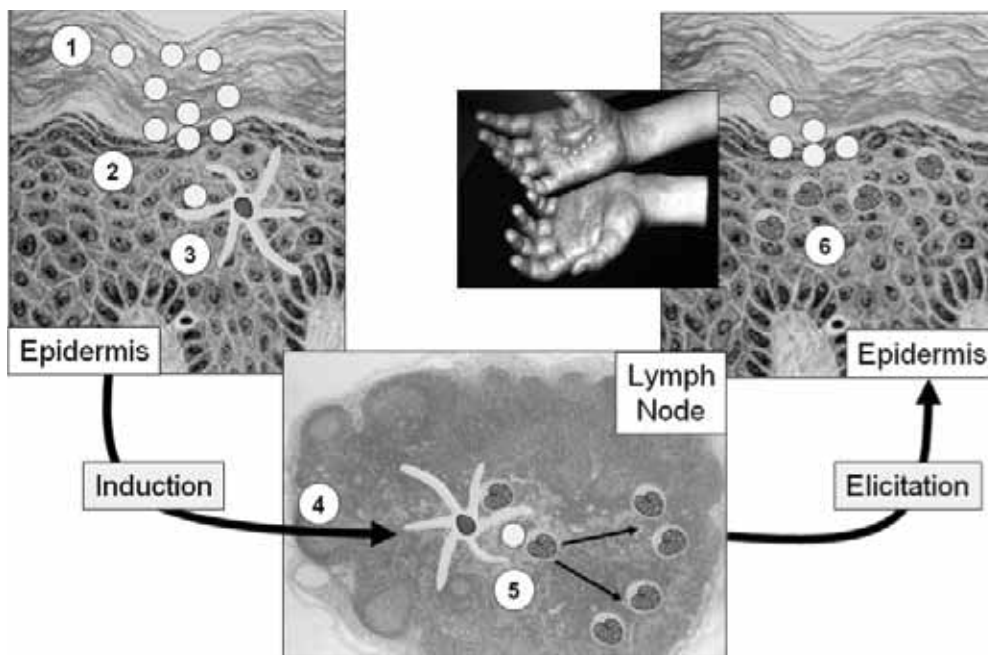
Figure 1. Mechanistic overview of induction and elicitation of allergic contact dermatitis. ACD is a complex, multi-stage process with two distinct phases: induction (step 1-5) & elicitation (6). Significant research activities over the past 20 years have ensured the key pathways driving the induction of skin sensitisation are relatively well characterised, they are as follows: step 1) skin bioavailability (i.e., diffusion of sensitising chemical into the viable layers of the skin); 2) protein binding (i.e., sensitiser modification of skin protein); 3) skin inflammation; 4) dendritic cell maturation; 5) T cell response (i.e., clonal expansion of T cells are specific for sensitiser-modified skin peptides). Following a significant increase in the number of sensitiser-specific T cells in the body, subsequent exposure of the skin to the sensitising chemical will elicit an inflammatory response (step 6).

by the induction of receptor and cytokine-mediated co-stimulatory signals. If all of these signals are appropriately delivered, then T-cell proliferation will increase the frequency of hapten-specific T-cells. Upon reaching an unknown threshold number of hapten/peptide-specific T-cells, an individual will be said to be 'sensitised' and will elicit an inflammatory response upon re-exposure to the sensitiser. These eczematous skin reactions (termed ACD) are driven by hapten-specific CD4+ T-helper 1 (Th1) and CD8+ cytotoxic T-cells, and

have been shown to increase in severity upon each subsequent re-exposure. Several non-animal test methods have been developed to characterise these key toxicity pathways[20-23]; however, developing a mechanistic rationale to allow the interpretation of these non-animal data still represents a major challenge.

Jowsey et al.[24] initiated a discussion on a weight-of-evidence approach to the integration of several key processes known to be important mechanistically

in the induction of skin sensitisation. The approach is based upon scoring and integrating the outputs from a series of different non-animal hazard methods (i.e., structural alert, bioavailability, protein binding, DC maturation, and T-cell proliferation) to generate a new measure of skin sensitisation potency[24]. This is a pragmatic 'weight-of-evidence' approach to data integration; however, our current view is that mathematical modelling approaches will be required to integrate and interpret test method data in a more mechanistically-relevant way. To test and explore the relative contributions of individual biological pathways thought to be key to the induction of skin sensitisation, we developed an *in silico* mathematical model (termed the Skin Sensitisation PhysioLab® (SSP) platform[25]) in collaboration with Entelos® Inc.

The SSP platform was constructed to capture our current understanding of the pathways involved in the induction of skin sensitisation by using the published literature to define and calibrate the biological relationships[25]. The information used to build and define the biological relationships is accessible and can be replaced with new information as it becomes available. Consequently, the model is not viewed as an exhaustive record of all the biological pathways involved in the induction of skin sensitisation. It represents the current best hypothesis of the pathways known at present to be required (i.e., quantitative data exist in the literature to support their roles). The development of the model required extensive quantitative data analysis at the

level of individual pathways, as well as whole-scale system dynamics. Calibration experiments were performed to ensure that the key biological mechanisms and dynamics of individual pathways were consistent with the published data. Several evaluation experiments were performed, to ensure that the individually calibrated pathways, when acting together in a system, reproduced the dynamics observed experimentally, without any changes to the biological representation.

The results show that the calibrated and evaluated SSP platform reproduced many of the key experimental benchmarks observed in several studies[25]. Data on dLN cell numbers and composition, as well as on antigen-specific T-cell proliferation, were used to create reasonable ranges that defined an acceptable response. Furthermore, the platform ultimately reproduced the key dynamics observable with the LLNA, and also allowed exploration of the underlying mechanisms giving rise to the observed phenomena (often difficult to measure *in vivo*), such as the effect of sensitiser dose on the time at which the sensitiser-specific T-cell proliferation response is maximal. In addition the approach also provided new biological insights through combining previously-disparate data sets and drawing parallels from other disease processes[25].

We also conducted a sensitivity analysis on the model, as a first step in determining the relative contributions of individual pathways to the induction of skin sensitisation[25]. The output of primary interest for the assessment of

sensitiser potency was the ability to induce CD4$^+$ and CD8$^+$ Ag-specific T-cell proliferation. Therefore, we measured the impact of several pathways on this output variable, and explored sensitivities for prototypical weak, moderate and strong sensitisers. Reassuringly, not one but several key pathways were found to drive the sensitiser-specific T cell response, and these pathways could be broadly categorised into the following groups: skin bioavailability; protein reactivity; skin inflammation; DC maturation (including DC activation in skin and DC migration to dLN); T cell proliferation. This analysis has been used to focus our fundamental research studies, evaluate the relative predictive power of different *in vitro* test methods and aid the development of a mechanistic rationale for the interpretation and integration of non-animal hazard information[25].

New Metrics for Skin Sensitisation

Based upon our evaluation of the SSP platform, we submit that one or more of the following categories of non-animal information should, when combined via mechanistic, computational modelling, yield a robust prediction of the hapten-specific human T cell response for a given skin exposure to sensitiser: protein reactivity; skin bioavailability; skin inflammation; DC maturation; T cell proliferation. Examples of our current research, and related research being carried out by others into the development and application of non-animal predictive methods, are outlined below.

1. Protein Reactivity

It is generally understood that any chemical (or a metabolite of it generated within the skin) must form a stable (covalent) adduct with protein in the skin in order to stimulate an immune response [26-28]. Consequently, the covalent modification of a protein by a reactive chemical (haptenation) is considered to be a key step in the induction of skin sensitisation. The ability of a chemical to sensitise is intrinsic in the properties of the chemical itself. Several *in chemico* assays for measuring the extent and nature of chemical reactions with model peptides are being developed, underpinned by this hypothesis [20,29-31]. For the purposes of deriving the maximum qualitative and quantitative information on the reactivity of a chemical with peptides, we have developed an *in chemico* peptide reactivity profiling assay, which uses a panel of six single-nucleophile peptides (generic sequence AcFAAXAA, where X = Cys, Lys, Tyr, His or Arg, with H$_2$N-FAAAAA representing the N-terminal nucleophile), with the aim of determining the reactivity profile of a chemical with a high level of confidence. In principle, the reactivity profiling in this assay is similar to that in previously-published methods[29,30,32]. Each peptide is incubated with the test chemical under conditions optimal for peptide reactivity (conditions which are not necessarily physiological). The main differences are the number of nucleophiles studied, and the addition of the adduct observation as a necessary step to distinguish a reactive chemical from non-reactive chemical. This is particularly useful in confirming whether the chemical

has reacted, when the peptide depletion values are small and within the variability of negative control (<15%). In addition to measurements of unchanged peptide and observation of the formed adduct(s), this approach permits the determination of reaction mechanisms[33], specificity, and relative rates of reactions [34]. The challenge going forward is whether it is possible to develop a mechanistic rationale for interpreting and integrating these data in the context of how much 'modified skin protein' would be generated following a given skin exposure to a specified sensitising chemical.

2. Skin Bioavailability

In order to induce skin sensitisation a chemical must first gain access to the viable layers of the skin with the most likely target site being the viable epidermis where immature DCs (termed Langerhans' cells (LCs)) reside. The amount of chemical available to the epidermis and the length of time that it is exposed depends on the rate of penetration, the distribution profile within the epidermis, and the removal of the chemical either by metabolic or dermal capillary clearance. The epidermal bioavailability of chemical can be summarised using kinetic models, yielding parameters such as the C_{max} (the maximum concentration in the epidermis), t_{max} (the time at which C_{max} occurs) and AUC (the area under the curve, a composite measure of the extent and duration of exposure). A new experimental approach based on *ex vivo* human skin has recently been investigated, whereby such parameters can be estimated for

the epidermis and dermis following topical exposure[35,36]. These experiments examined the effects of physiochemical parameters such as lipophilicity, volatility and vehicle on the chemical kinetics of skin bioavailability. However, the bioavailability of free chemical in the skin tissue is also influenced by skin metabolism, tissue adsorption and clearance mechanisms, and a previous literature review [37] has revealed a lack of fundamental knowledge about human skin metabolism. The *in vitro* and *ex vivo* characterisation of skin metabolism is challenging due to the loss of metabolic function almost immediately following biopsy. Nevertheless, if experimental methods could be developed that measured the metabolic clearance (or activation) of skin sensitisers, this information could be combined with data on penetration kinetics and protein reactivity to determine bioavailability of modified skin protein in the epidermis.

3. Skin Inflammation

Keratinocytes are the predominant epidermal cell type within the skin. They are known to produce large quantities of inflammatory cytokines, which serve to alert LCs to the presence of a pathogenic infection or physical/chemical stressor[38]. There have been significant recent efforts to develop *in vitro* test methods based upon measurement of chemical-induced changes in cell stress pathways in the HaCaT human keratinocyte cell line[39,41]. The development of KeratinoSens[40] and SenCeeTox[41] test methods built upon the observation that sensitisers appeared to preferentially activate the Nrf-2 cell stress

pathway when evaluated using the AREc32 cell line[39]. Although such advances in test method development are impressive there is still a need to understand whether sensitiser-induced changes in these cell stress pathways simply confirm the inherent chemical reactivity of the sensitiser (section 1 above) or whether they provide additional information.

4. DC Maturation

Independent of their interactions with keratinocytes, the phenotypic changes that LCs/DCs undergo upon sensitiser exposure have been extensively studied over the last 20 years and form the basis for several DC-based predictive methods[22,42,43]. Two DC maturation test methods currently under evaluation, the h-CLAT[44] and the MUSST test[45] are based upon the measurement of relative changes in cell surface receptor expression (CD54 and CD86 in the h-CLAT test, and CD86 in the U937/CD86 test). In addition, the VITOSens approach developed by Hooyberghs et al.[46-47] used an algorithm to interpret changes in key genes following exposure of a cord blood-cell derived DC model to the chemical of interest.

Clearly a mechanistic rationale can be postulated for the inclusion of skin inflammation and DC maturation test methods in a non-animal toolbox for skin sensitisation; however we have yet to identify a need for these data when modelling the hapten-specific T cell response.

5. T Cell Proliferation

Naïve T cell proliferation in response to chemical treatment is a robust indicator that a substance is immunogenic, and several publications are concerned with demonstrating the experimental feasibility of inducing a naïve T-cell proliferation *in vitro* following co-culture with chemical sensitiser-treated DCs or LCs[48-49]. However, the sensitivity of these approaches has not been demonstrated to date, as significant proliferative responses have generally only been detectable following stimulation with sensitisers of strong potency. From our own experience, the complexity of DC:T-cell co-culture protocols make these approaches both labour intensive and difficult to standardise. Furthermore, our SSP platform analysis of dLN cell dynamics has highlighted the importance of LN trafficking in driving the sensitiser-specific proliferative response[25], i.e. a dynamic system is needed. Such trafficking will be difficult to reproduce *in vitro*, due to the static nature of the standard DC: T cell co-culture methods. Therefore, we are keen to explore the apparent benefits of modifying these protocols to exclude the regulatory T cell fraction[50] and to investigate the potential benefits of novel tissue engineering technologies[51].

In May 2010, Unilever held a workshop (London, UK) to explore the relationship between sensitiser-induced T cell responses and sensitiser potency with experts from various disciplines including immunology, mathematical modelling, and risk assessment (Kimber et al.; manuscript in preparation). After 2 days

of presentations and discussions, the key conclusion was that our mechanistic understanding of how the sensitiser-induced T cell response relates to sensitiser exposure in humans is significantly lacking and is based upon the assumption that sensitisers and pathogens (e.g., herpes virus) induce adaptive immune responses via the skin in a mechanistically-similar way. Clearly there will be a need to confirm or challenge this assumption over the coming years through working more closely with clinical immunologists and dermatologists working in the field.

Discussion

Assuring consumer safety without animal testing represents a formidable challenge. During the last 10 years, *in vitro* test methods have been adopted to replace the need for animal testing for several consumer safety endpoints (e.g., skin corrosion, phototoxicity and skin irritation). The development of these methods was primarily driven by the need to identify hazard, with the end result that the animal method is replaced with one non-animal method[52]. However, for repeat-dose endpoints, the expectation that full replacement can be achieved through the development of a single non-animal method is now widely believed to be unrealistic[24,53-54]. Consequently, it is now increasingly accepted that toolboxes of non-animal test methods (each aligned to different toxicity pathways) and mechanistically-relevant computational models (capable of integrating these non-animal test data) will be required to predict the likelihood of an adverse or non-adverse event (e.g., induction of skin sensitisation) occurring for a given exposure scenario. Thus, we are witnessing a paradigm shift in how 'replacement' will be achieved going forward.

Next Steps

In recent years we have significantly increased our knowledge of the toxicity pathways driving the induction of skin sensitisation; however we still lack sufficient mechanistic understanding to relate sensitiser exposure to any aspect of the hapten-specific T cell response (e.g., how does high sensitiser reactivity to skin protein relate to the magnitude, quality or breadth of the T cell response?). Consequently going forward we have focussed our research efforts on developing a mechanistic computational model of the induction of skin sensitisation that can relate a 'total modified skin protein' metric to one or more key metrics of the sensitiser-induced T-cell response. The computational model will be underpinned by fundamental research activities to increase our understanding of key toxicity pathways and their impact on the adverse immune response. In addition, new non-animal test methods will be developed and existing test methods optimised to provide relevant information to inform model predictions for given chemicals and exposure scenarios. Finally, human-relevant *in vivo* benchmarks will be established through relevant clinical studies to replace our current dependence on sensitiser potency predictions derived from historical LLNA studies.

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Marker Discovery & Their Functional Role in the Adverse Outcome Pathway for Skin Sensitisation

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Background & Objectives

European legislation and animal welfare organisations have stimulated research to develop non-animal toxicity tests. Assays for identifying skin sensitisers are highly warranted since all chemicals with annual production volumes above 1 tonne should be tested for this endpoint (Schoeters, 2010). VITO-CARDAM is involved in evaluating adverse health effects of chemicals in relation to allergies. As such, over the years, an assay has been developed with the intention to mimic as closely as possible the human response to skin sensitisers. The assay is based on primary cells of human origin. CD34+ progenitor cells are isolated from human cord blood and differentiated *in vitro* to dendritic cells (DC), which exhibit characteristics of the professional antigen-presenting cells of the skin (De Smedt et al., 2001). DC are recognised as cellular players in the induction phase of sensitisation and are main actors in the adaptive immune response cascade (Steinman, 2001). Our *in vitro* research focused on understanding the cellular and molecular changes in DC after exposure to chemical sensitisers. This mechanistic approach is much in line with the National Research Council (NRC) vision for chemical safety assessment, which highlights the need of alternative tests for cellular responses and toxicity pathways with a key function in the development of adverse health effects in humans (Collins et al., 2008).

Although the mechanisms underlying skin sensitisation are not yet fully understood, some aspects are well known. For instance, upon hapten encounter, DC undergo the maturation process during which their phenotypical appearance changes in order to permit their migration to the local lymph nodes and activation of surrounding cells (Romani et al., 2003). These transitions are characterised by the altered expression of cell surface markers on DC and secretion of cytokines. Similar changes occur after exposure of *in vitro* differentiated DC to chemical skin sensitisers. We could correctly classify 9 skin sensitisers and 2 irritants after 24 h exposure of *in vitro* differentiated DC based on changes in surface marker expression (CD86, CD83 and HLA-DR) (De Smedt et al., 2005). All allergens tested induced a significant increase in at least one of the DC surface markers. In contrast, none of the irritants tested were able to significantly up-regulate membrane marker expression in exposed DC. We concluded that the *in vitro* CD34-DC model has the capacity to distinguish between chemical sensitisers and irritants based on altered phenotypic characteristics. However, the dynamic range of the response is limited (Casati et al., 2005). More recently we showed stimulation of CD83 and CD86 by the endotoxin Lipopolysaccharide S (LPS), which is considered to be a sensitising adjuvant but not a sensitiser itself (Lambrechts et al., 2011).

DC maturation is characterised by changes in surface membrane markers of DC, but also by changes in cytokine secretion. Several studies turned these molecules into possible biomarkers and tested their discriminating power (dos Santos et al., 2009). In the CD34-DC model, secretion of cytokines such as IL-1β, IL-6, IL-12 and TNF-α were studied. These markers showed differential expression levels after exposure to a limited set of sensitisers, but changes in cytokine production were not consistently related with exposure to an allergen (De Smedt et al., 2001).

Based on the above-mentioned findings, we applied a new, holistic approach using microarray technology. The genome of DC was screened for differential expression after *in vitro* exposure to 4 skin sensitisers and 2 non-sensitisers. This resulted in the selection of a set of 13 genes as novel potential biomarkers (Lambrechts et al., 2011). The discriminating potential of these markers was confirmed by qPCR experiments on an extended data set of 21 chemicals. A final classification model based on 2 genes, CCR2 and CREM, could be built that predicts the sensitising identity of a chemical with 89% concordance, with a sensitivity and specificity of 82% and 97%, respectively (Figure 1) (Hooyberghs et al., 2008). This prediction assay was named VITOSENS™. The sensitising adjuvant LPS, which shows a stimulatory effect on surface membrane markers of DC, did not affect the expression of CCR2 and CREM what confirms the specificity of the VITOSENS™ assay.

The expression analysis of these new molecules at the gene level offers a new source of information on the skin sensitisation pathway. Furthermore, the de-

tection method, qPCR, is a reliable and quantitative technique that can assess multiple markers in parallel and can easily be transferred to a high-throughput protocol. This new lead in the VITOSENS research is in line with the US NRC vision of 'toxicity testing in the 21st century' (TT21c), because it puts emphasis on the elucidation of toxicity pathways by means of high-throughput screens conducted in human tissue (Andersen & Krewski, 2009; Xia et al., 2008). To further implement this vision, we explored the molecular mechanism by which VITOSENS™ responds to

skin sensitisers. Gaining more knowledge on the mode-of-action of sensitisers in *in vitro* assays will further supports the use of these assays for human safety evaluation.

Goals & Achievements

Towards Mechanism-Based Predictions

When an *in vitro* assay incorporates the complex, underlying molecular events of
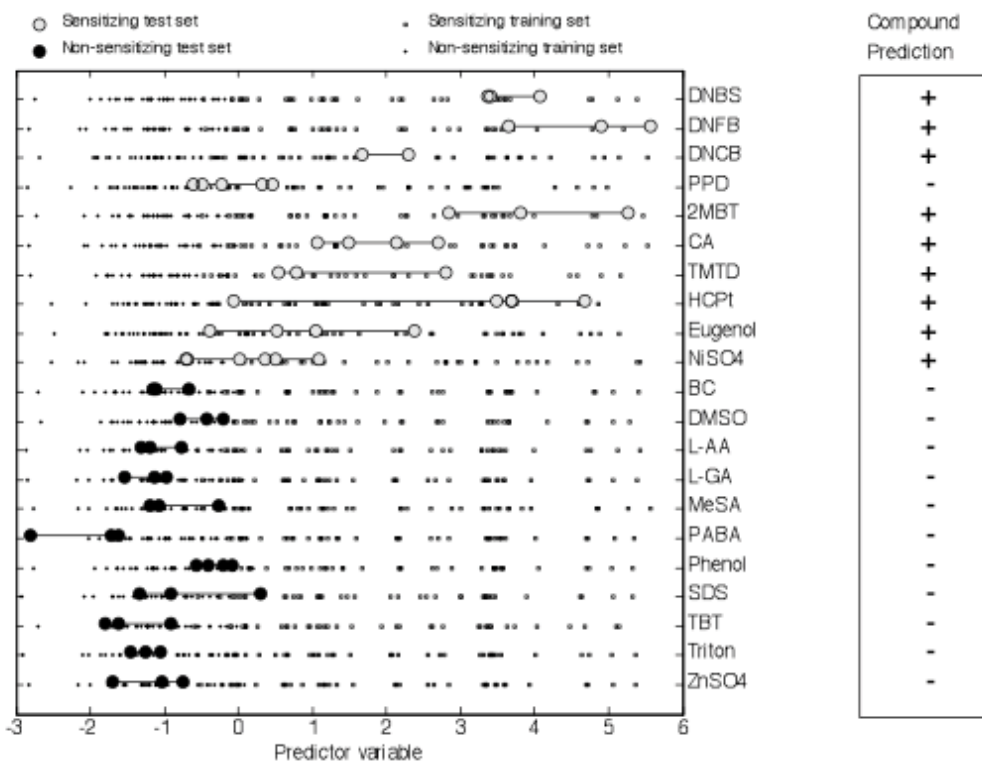
Figure 1. Predictions of the VITOSENS® model in the cross-validation (Hooyberghs et al., 2008). Per test compound, each circle corresponds to a donor sample and is represented by its predictor value: positive = sensitised, negative = non-sensitised. The right panel gives the prediction per compound by applying a majority rule on the representing test samples: + = sensitiser, - = non-sensitiser. Prediction is based on human data and on results from the local lymph node assay.

skin sensitisation, it will aid in a better extrapolation of data and an improvement of human hazard identification. To this end, we evaluated the involvement of the marker genes in the skin sensitisation process.

A molecular network constructed around the VITOSENS™ gene markers using data-mining software showed a relation with other molecules that are associated with the skin sensitisation process. CREM and COX2 showed a central role in the generated network and additional molecules were identified as potential regulators in skin sensitisation pathways (Figure 2). qPCR experiments confirmed gene expression of a selected number of these network members in CD34-DC after sensitising chemical exposure. The relevance of some of the VITOSENS™ marker genes was further evaluated by analysing the expression of corresponding proteins. The protein expression pattern of the discriminating marker CCR2 and network regulator COX2 was similar to the differential gene expression after exposure of DC to skin sensitisers. Recently published observations on VITOSENS™ confirm a functional role of these molecules in the sensitisation process since modulation of CCR2 expression significantly altered HLA-DR surface expression on DC, while inhibiting COX2 resulted in the down-regulation of co-stimulation marker CD86 (Lambrechts et al., 2011).

Applying In Vitro Hazard Markers for Potency Characterisation

The above-mentioned insights aided in the design of a prediction model for skin sensitising potency *in vitro*.

Although useful for dichotomous classification of chemicals as being a sensitiser or not, the gene expression fold changes (FC) obtained in VITOSENS® yielded insufficient information to rank the chemical sensitisers according to their potency. However, by combining both the FC and the concentration of the sensitiser that yielded 20% cell damage (IC20) in a robust multiple linear regression analysis, a potency value was modeled that closely fitted *in vivo*-derived (LLNA) potency data, and this over the entire range from weak to extremely sensitising chemicals (Figure 3) (Lambrechts et al., 2010b). This shows that in order to rank skin sensitisers according to their potency, the chemical's inherent danger capacity should be taken into account as well. In general, the lower the concentration of the chemical to induce a defined level of cellular damage, the more potent it is for sensitisation induction. We can speculate whether concurrent application of irritants and sensitisers would lower the threshold of response for a given sensitiser. Similar findings have been observed *in vivo*; concurrent application of allergens and irritants promotes a stronger clinical response than when applied separately (Grabbe et al., 1996; Pedersen et al., 2004).

The feasibility of classifying sensitisers into several potency classes based on *in*

vitro data may contribute to human risk assessment. Potency information may allow derivation of threshold concentrations that are required for the induction of skin sensitisation. Optimising this prediction model may generate more robust data on the potential risks to humans posed by exposure to environmental agents and may
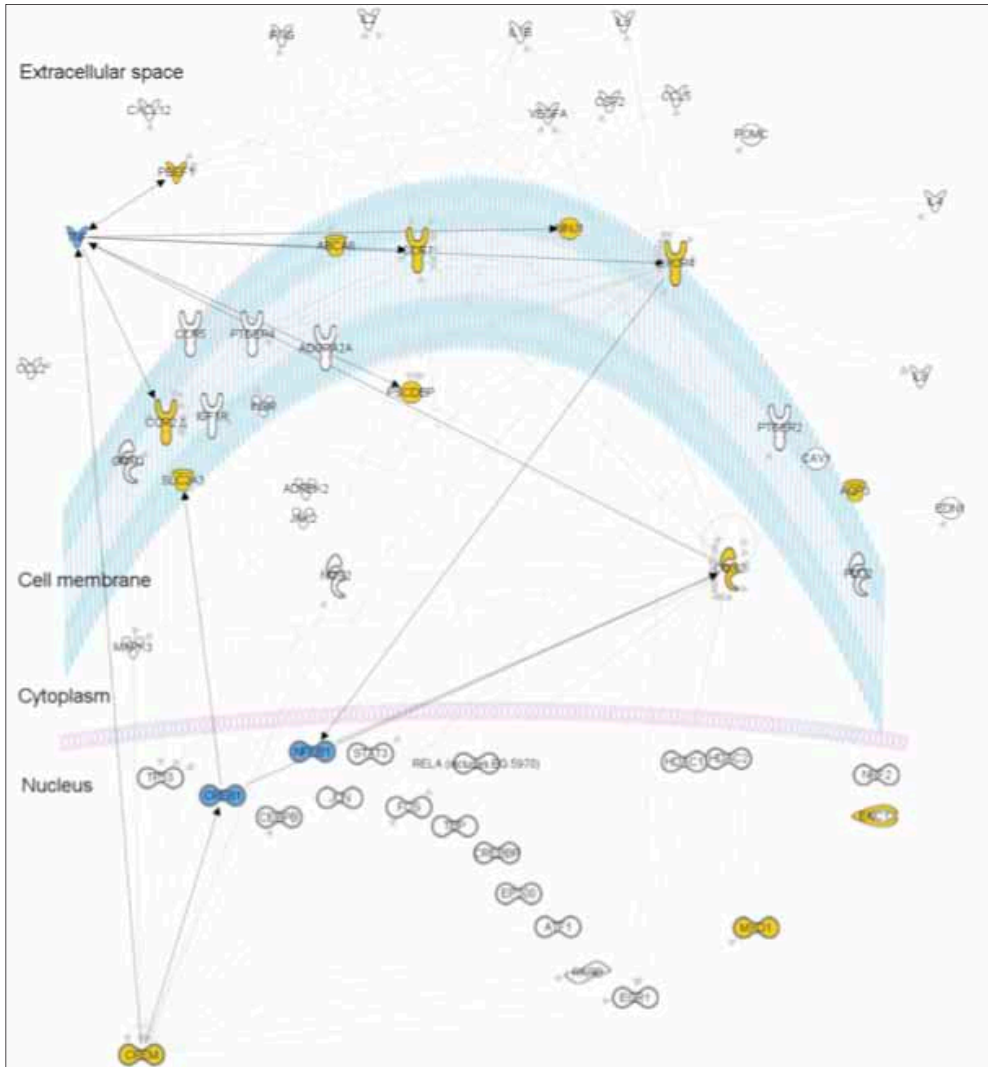
Figure 2. Human cellular network comprising 13 VITOSENS® biomarkers (Lambrechts et al., 2010). Thirteen VITOSENS® biomarkers (yellow) were loaded into the Ingenuity Pathways Analysis software. Forty-four molecules were added to connect the 13 biomarkers of which 20 were located in the nucleus, 7 in the cytoplasm, 15 in the plasma membrane and 15 extra-cellular. Three molecules (CREB1, NF-$_K$B1 and TNF-α, in blue) were selected for confirmation of mRNA expression in (non-) sensitiser exposed CD34-DC. The direction of the arrow indicates the direction of influence the molecules act upon each other.

Figure 3. Scatter plot of robust linear fit results from *in vitro* data (IC20 and fold-changes) versus in vivo EC3 (Lambrechts et al., 2010). The dashed line is the identity diagonal, added as a visual guide. Per compound the mean result of at least 3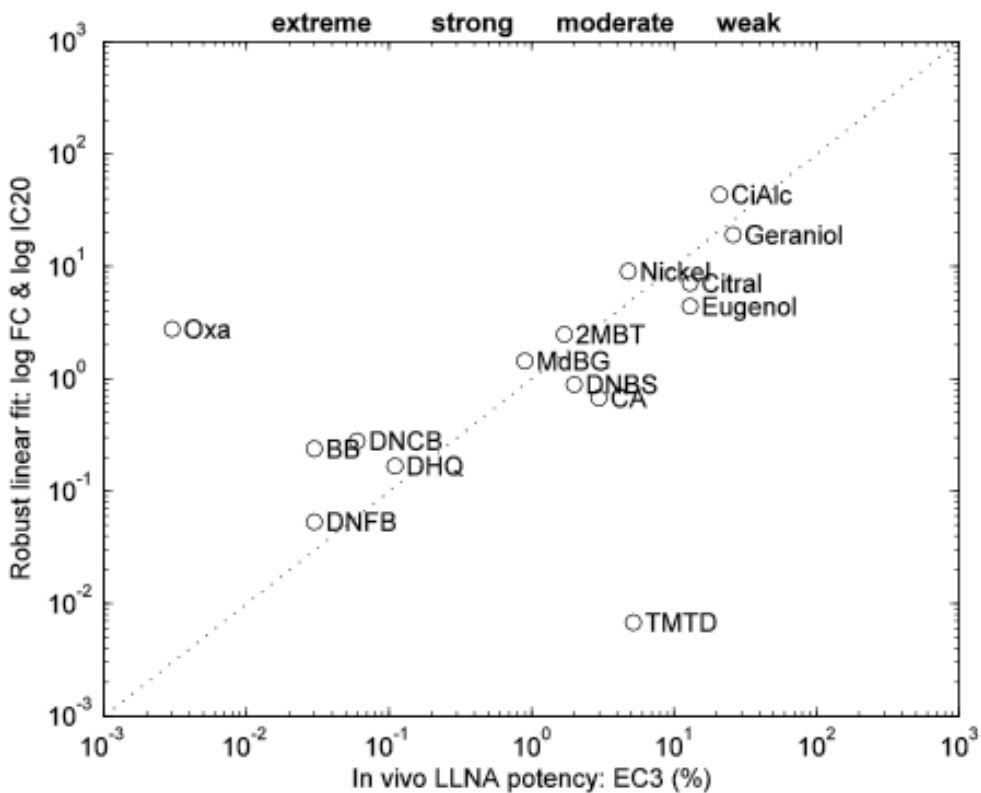 replicate measurements from separate donor samples is shown. After removal of the outlying compounds, Spearman rank-correlation coefficient = 0.91, and Pearson correlation coefficient = 0.79. The 4 subcategories for LLNA potency classification are indicated on top of the graph.

expand capabilities to test chemicals more efficiently. A stronger scientific foundation offers the prospect of improved risk-based regulatory decisions and possibly greater public confidence in and acceptance of the decisions.

Next Steps

Since *in vitro* assays only cover a specific step in the complex biological cascade of skin sensitisation as it occurs *in vivo*, it is unlikely that a single method will be able to substitute the complex human response on skin sensitising chemicals (ECHA, 2008). Instead of dissecting all elements of the immunobiological response of skin sensitisation using distinct tests, recently a strategy for the integration of the various data elements has been proposed (Basketter & Kimber, 2009; Jowsey et al., 2006). Each building block in such an integrated approach should meet criteria

for entering pre-validation, including its mechanistic relevance, predictive capacity and evidence of the reproducibility of the method (ECHA, 2010). As such, human DC responses will contribute to hazard and potency knowledge of chemicals. In combination with other datasets the current prediction models for sensitisation will be improved. The use of primary human cells would even allow the study of the heterogeneous response in the human population.

Although the molecular approach of VITOSENS™ has been a step forward, further challenges are to increase throughput of the assay allowing generation of testing results more rapidly and at lower costs, thereby deriving dose-reponse curves. This is essential information for obtaining dose thresholds (no expected sensitisation induction level (NESIL)). We are still collecting information on metabolic capacity of the assay. To delineate the application domain of VITOSENS and evaluate its response to chemical mixtures remains another challenge.

The European REACH regulation as well as the US NRC TT21c vision offer new perspectives for intelligent chemical safety assessment. They put emphasis on human risks, on reduction of the use of animals and on incorporation of mechanistic information in a weight-of-evidence approach (REACH) or a translational approach (US vision). Model systems that reveal the mode of action of chemicals and elucidate human disease pathways will ultimately lead to improved human safety assessment (Schoeters, 2010).

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The OECD Adverse Outcome Pathway Approach

A Case Study for Skin Sensitisation

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Introduction

Development and implementation of more integrated approaches to assessment of organic chemicals is a challenge facing many governing bodies. The challenge is to develop a process which shifts, in a scientifically credible and transparent manner, from a paradigm that requires extensive *in vivo* testing to a paradigm that provides a hypothesis-driven approach which identifies specific information, especially *in silico*, *in chemico* and *in vitro*, most relevant to the particular assessment. The goal is to require only those *in vivo* tests that are critical to addressing the remaining uncertainties in the assessment.

One approach to meeting this challenge being examined by the Organisation for Economic Co-operation and Development (OECD) is the 'adverse outcome pathway' (AOP). An AOP represents existing knowledge concerning the linkages between a

molecular initiating event and an adverse outcome at the individual or population level (OECD, 2011a). As such, an AOP, by definition, has a single defined molecular initiating event and a stated *in vivo* hazard outcome but spans multiple levels of biological organisation. As will be demonstrated in the example for skin sensitisation, AOPs describe the casual linkages between chemical structure and the initial molecular interaction (e.g., protein binding) and the sequent cellular responses (e.g., gene activation and protein expression), organ-level responses (e.g., T-lymphocyte proliferation), and ultimately an *in vivo* adverse outcome (e.g., chemical allergen).

Different data that inform the AOP include, for example; structural alerts that are reflective of the types of chemicals that can initiate a pathway, *in chemico* methods that measure the relative reactivity or chemical-biological interactions, *in vitro* assays that confirm the sequent cellular responses (e.g., gene expression), and ultimately, *in vivo* tests that measure endpoints that are directly relevant to the adverse outcome that drive regulatory decision making. By understanding the likelihood of effects at the chemical level and/or lower levels of biological organisation from structure-activity relationships (SARs), and *in chemico* and *in vitro* assays, one could efficiently determine if additional tests at higher levels of biological organisation (e.g., *in vivo* assays) are required.

The AOP and mode of action (MoA) are similar concepts. Both define the sequence of key events, starting with the interaction of an agent with the target cell through functional and anatomical changes, to *in vivo* adverse effects of regulatory interest (Boobis et al., 2008). The MoA framework described by Boobis et al. (2008) is designed to determine human relevance of animal data and as such, it requires a more detailed understanding of both the pharmacodynamic phase and pharmacokinetic phase of toxicity, as well as interactions between the two phases. It also provides a weight-of-evidence approach that is based on considerations for causality as originally articulated by Hill et al. (1965).

The development of AOPs includes fundamental aspects of the MoA framework, including the sequence of key events and consideration of the weight-of-evidence for these events. In the OECD's AOP approach, once the AOP is established, data for the molecular initiating event and other key events can be used for chemical category formation. A chemical category is a group of chemicals whose properties are likely to be similar or follow a regular pattern because of their similar chemical structure. Using this so-called category approach, not every chemical needs to be tested for every endpoint because the available test results for the members of the category allow an estimation of the results for the untested endpoints (OECD, 2007). Data gaps to be filled by read-across from tested chemicals within the category to untested chemicals within the same category. Using the AOP approach, chemicals can be grouped according to the pathway that they are triggering. Depending on the decision being made,

the AOP can be used in qualitative or quantitative scenarios. The premise being that if a new compound elicits the molecular initiating event (and other key events) it will potentially produce the adverse effect defined by the AOP.

In the OECD approach, it is considered critical to be able to gauge the reliability and robustness of an AOP; this is done by evaluating the experimental support of the AOP. Included in this evaluation is:

a) an assessment of the qualitative understanding of the AOP
b) an assessment of the experimental evidence or data
c) a statement of confidence in the AOP
d) an assessment of the quantitative understanding of the AOP.

Results

Skin sensitisation is a well-studied toxic endpoint of which aspects have been the subject of many scientific experiments over the past decade. Skin sensitisation is a Type IV contact allergy which is typically described in two phases— the induction of sensitisation and the subsequent elicitation. The first phase includes a sequential set of events that are described in this AOP. While there is generally agreement regarding these events (Karlberg et al., 2008; Aeby et al., 2010; Adler et al., 2011), understanding of the underlying biology of many of the key events remains incomplete. Because of biological complexity (e.g., multiple organs and multiple cell types), skin sensitisation is evaluated with *in vivo*

tests but alternative methods are under development (Aeby et al., 2010).

While non-covalent reactions with metals and redox cycling have been linked to skin sensitisation, the AOP described here is designed to address organic chemical agents that react with thiol (i.e., cysteine) and primary amines (e.g., lysine). The earlier work on the molecular basis of skin sensitisation was reviewed by Lepoittevin et al. (1998). However, knowledge on the key events associated with skin sensitisation has evolved rapidly over the past decade. This knowledge may be summarised as:

1) The target substance must be bioavailable (i.e., it must penetrate the stratum corneum of the skin).
2) The target substance must be a direct-acting electrophile, be converted from a non-reactive substance (pro-electrophile) to a reactive metabolite via metabolism, or be converted from a non-reactive substance (pre-electrophile) to a reactive derivative via an abiotic process, typically oxidation.
3) The molecular sites of action are targeted nucleophilic sites in proteins (e.g., cysteine and lysine residues) in the dermis.
4) The molecular initiating event is the covalent perturbation of dermal proteins, which is irreversible (i.e., formation of the hapten-protein complex or complete antigen). *In vivo* this event is associated with the production of a specific memory T-cell response.
5) Biochemical pathways affected by the definitive electrophile's action on the

molecular targets are incompletely known but often include the mitogen-activated protein signaling pathway and the oxidative stress response pathway, especially in keratinocytes.

6) The cellular-level outcomes are incompletely known but include immune recognition of chemical allergens by Langerhans cells (specialised epidermal dendritic cells) and dermal dendritic cell. Responses in the form of expression of specific cell surface markers, chemokines, and cytokines are typically taken as evidence of dendritic cell maturation.

7) The organ-level responses include:
   a. dendritic cell migration to the lymph node, and
   b. differentiation and proliferation of allergen-specific memory T-cells.

8) The target organ(s) are the skin and required intact local lymph nodes; the target cell populations are the immune cells, especially T-cells.

9) The key physiological response is acquisition of sensitivity.

10) The key organism response is dermal inflammation upon receiving the substance challenge in the elicitation phase. This response is associated with stimulation of specific memory T-cell produced in the induction phase.

11) The overall effect on mammals is allergic contact dermatitis in humans or its rodent equivalent, contact hypersensitivity.

An assessment of the qualitative understanding of the AOP should include a listing of the key events, as well as the documentation of the experimental support for each event and an evaluation of the strength of scientific evidence for that event. For this case study the information is summarised in Table 1.

A second part of the OECD approach is to answer a series of questions designed to summarise the current experimental evidence supporting the proposed AOP. An AOP may be considered either plausible or probable, depending upon the extent (i.e., depth and breadth) of the available scientific evidence supporting the AOP, and the extent to which the key events have been experimentally tested and found to be consistent with empirical data. In this regard, an AOP can be thought of as an evolving entity. With regards to this case study for skin sensitisation, the following questions are asked and answered:

- *How well characterised is the AOP?* The skin sensitisation AOP is at least qualitatively well characterised, as the seminal events are generally accepted by the scientific community.
- *How well are the initiating, and key events causally linked to the outcome?* The molecular initiating event (protein binding reactions) is based on long-standing, well-studied organic chemical mechanisms and reactions. T-cell proliferation and to a lesser extent dendritic cell activation/maturation, as well as keratinocyte-based gene expression, are causally linked to sensitisation.
- *What are the limitations in the evidence in support of the AOP?* While there is general agreement that the AOP for

Table 1. Summary of the experimental evidence for the skin sensitisation AOP.

Key Events	Experimental Support	Strength of Evidence
Key Event 1 (initial event)	Site of action proteins (Karlberg et al., 2008; Wong & Liebler, 2008). Covalent binding at cysteine &/or lysine (Roberts & Natsch, 2009; Schwöbel et al., 2011)	Strong; well accepted mode of toxic action associated with skin sensitisation (Gerberick et al., 2008; Aeby et al., 2010); 100's of chemicals evaluated for binding; quantitative end-points
Key Event 2	Gene expression of antioxidant response element in keratino-cytes (Natsch & Emter, 2008; Emter et al., 2010; McKim et al., 2010)	Strong/Adequate; well accepted cell signalling pathway associated with skin sensitisation; 10's of compounds evaluated; quantitative endpoints
Key Event 3	Activation of dendritic cells (dos Santos et al., 2009; Vandebriel et al., 2010; Ashikaga et al., 2010)	Adequate; well accepted expressions of co-stimulatory molecules & cytokines associated with skin sensitisa-tion; various endpoints; 10's of compounds evaluated; tend to be qualitative rather than quantitative
Key Event 4	T-cell proliferation (Gerberick et al., 2005; Kern et al., 2010)	Strong; two decades of development & testing with the Local Lymph Node Assay (LLNA); 100's of chemicals evaluated; quantitative end-point
Adverse Outcome	Allergic contact dermatitis in humans or its rodent equivalent contact hypersensitivity	*In vivo* data sets for human & guinea-pig

skin sensitisation outlined above is appropriate for qualitative hazard identification, there is no agreement on what measurements, other than reactivity, are necessary to reflect potency.

- *Is the AOP specific to certain life stages / age classes (i.e., are there critical life stages where exposure must occur to result in the adverse effect)? Or, is the AOP known to be initiated regardless of life stage but key events along the*

pathway are different dependent on life stage? The proposed skin sensitisation AOP is not associated with life stage·, sex· or age·dependency.

- *Are the initiating and key events expected to be conserved across taxa?* While *in vivo* testing focuses on selected mammals, including humans, the key events are conserved across mammals.

An additional aspect of the OECD approach to using the AOP concept is to implement the Hill criteria (Hill, 1965) to assess the weight·of·evidence supporting the AOP. With regards to the case study for skin sensitisation, the following issues are address:

- *Concordance of dose·response relationships:* While no specific citations were found, there appears to be general agreement among the dose·response relationships (both within and between assays) for both skin sensitisers and non·sensitizsers tested in mice, guinea pigs, and humans. The major problem is that earlier results were not intended to be compared quantitatively.
- *Temporal concordance among the key events and adverse outcome:* There is good agreement between the sequences of biochemical and physiological events leading to skin sensitisation (Gerberick et al., 2008; Karlberg et al., 2008, Alder et al., 2011).
- *Strength, consistency, and specificity of association of adverse outcome and initiating event:* There is excellent strength, as well as good consistence

and high specificity, of the association between *in vivo* skin sensitisation and *in chemico* protein binding. This is especially true for reactions that have thiol as the preferred molecular target (Schwöbel et al., 2011). There is excellent interlaboratory/protocol correlations within and between nucleophile depletion and adduct formation methods (Schwöbel et al., 2011).

- *Biological plausibility, coherence, and consistency of the experimental evidence:* The *in chemico*, *in vitro*, and *in vivo* experimental evidence is logical and consistent with the mechanistic plausibility proposed by covalent reactions based on the protein binding theory (Gerberick et al., 2008; Karlberg et al., 2008; Alder et al., 2011).
- *Alternative mechanisms that logically present themselves and the extent to which they may distract from the postulated AOP (it should be noted that alternative mechanisms of action, if supported, require a separate AOP):* While covalent reactions with thiol groups and to a lesser extent amino groups, are clearly supported by the proposed AOP, reactions targeting other nucleophiles may or may not be supported by the proposed AOP.
- *Uncertainties, inconsistencies and data gaps:* Uncertainties include the structural and physico·chemical cutoffs between theoretical and measured reactivity (Schwöbel et al., 2011), the significance of the preferred amino acid target (e.g., cysteine versus lysine) (OECD, 2011b),

the significance of type 1 versus type 2 cytokine secretion profiles (Hopkins et al., 2005), and what constitutes *in vivo* sensitisation.

Inconsistencies within the reported data are seen. There are differences in *in vitro* responses for highly similar chemicals (Natsch & Emter, 2008; McKim et al., 2010). There are differences within and between *in vivo* test results (see Annex C of the European Centre for Ecotoxicological and Toxicological Chemicals, 2010). Additionally, highly hydrophobic chemicals, which are *in vivo* sensitisers, are not active in aquatic-based *in chemico* or *in vitro* assays.

Data gaps: Based on the more than 50 chemical reactions associated with covalent binding to thiol or primary amine moieties (OECD, 2011b), *in vitro* data for keratinocyte, dendritic cell, and T-cell assays, as well as *in vivo* sensitisation data, is incomplete in that it does not cover the chemical spaces associated with many of these chemical reactions; *in chemico* data is also incomplete, especially for reactions that favor amino acid targets other than cysteine.

The final aspect of the OECD approach to using the AOP concept is an assessment of the quantitative understanding of an AOP. This includes the evaluation of the experimental data and models used to quantify the molecular initiating event and other key events. It also includes transparent determination of thresholds and response-to-response relationships used to scale *in chemico* and *in vitro* effects

to *in vivo* outcomes.

For skin sensitisation, a major hurdle is moving from a qualitative AOP to a quantitative AOP. One aspect to be resolved is that of the *in vivo* data with which to scale the response-to-response ratios. Because the Local Lymph Node Assay (LLNA) can directly quantify the adverse outcome (Basketter et al., 2009), public databases have recently been made available (Gerberick et al., 2005; Kern et al., 2010). LLNA results are often compared with results from alternative methods (e.g., Ashikaga et al., 2010; McKim et al., 2010). Such one to one comparisons may not be the best approach. As noted by Basketter et al. (2009), the LLNA is not without limitations, including variability between EC3 values or any other value (i.e., ECx) within isoreactive mechanistic classes.

Discussion

Development of the toxicological understanding expressed in an AOP, from the causal molecular initiating event and subsequent key events to the adverse outcome, will determine the relevant and required knowledge for the assessment. Existing data, in the form of *in silico* models, *in chemico* and *in vitro* diagnostic assays will inform the need for further targeted testing either at the *in vitro* or *in vivo* level. Databases developed using *in chemico* methods and *in vitro* assays have the potential to add a greater weight-of-evidence to chemical categories initially formed by prediction of the molecular initiating event and/or key events by

in silico models. As the potential for a chemical (or a group of chemicals) to produce toxicological effects is refined in the context of an AOP, fewer tests at higher levels of biological organization are required.

As indicated by Bauch et al. (2011), not all key events in an AOP may have to be satisfied in order to make an assessment. Justification of an AOP will involve consideration of the information concerning the molecular initiating event, other key events, and the apical outcome which is the basis of the assessment, as well as the weight-of-evidence for each event. What is considered sufficient justification of an AOP will be use-dependent, with a greater justification required for decisions with greater potential impact. For priority setting, consistency of output of predictive tools (e.g., *in silico* models) may suffice; however, for decisions supporting risk management, consistency across several levels of biological organisation including anchoring to the apical effects, will likely be required. Initially, assessments likely will be limited to hazard identification (i.e., qualitative AOPs); later as assays reflect key events and databases from these assays are established, more potency-related or quantitative AOPs will be developed.

In the case study for skin sensitisation, the AOP is depicted as a linear progression of events across levels of biological organisation that translate a molecular perturbation (protein binding) to an adverse outcome (skin allergy). However, it is well recognised that AOPs actually operate as networks within systems biology and various AOPs can share key events and interact with one another in a variety of ways.

AOPs include the fact that chemical interactions are at the molecular level and not at the whole animal level. Thus, adverse effects observed *in vivo* are the result of many biological responses, as well as the chemical structure of the toxicant. Hence, AOPs are designed to avoid mixing data from multiple mechanisms or molecular initiating events, which can cause the same *in vivo* outcome. Within the OECD QSAR Toolbox (here after noted as the 'Toolbox'; *oecd.org/env/existingchemicals/qsar*), this is accomplished by profiling chemicals to identify possible molecular initiating events and grouping chemicals that trigger the same molecular initiating event(s) and hence the same AOP(s). Data gaps are then filled by read-across or trend analysis (i.e., a simple QSAR) from *in vivo* databases. However, such direct linkages are not common among human health effects. Moreover, without a transparent description of a plausible progression of adverse effects at the different levels of biological organisation, it is difficult to reliably form chemical categories based on 2-dimensional structure and subcategories based on similarity in toxicological behaviour; two crucial aspects of the Toolbox. AOPs aid in resolving these problems by grouping chemicals based on both up-stream chemical and down-stream biological processes. AOPs shift the emphasis for category formations from just intrinsic chemical activity to chemical activity,

plus the key events that occur across the different levels of biological organisation.

Since the formation of the hapten-protein complex is the molecular initiating event for skin sensitisation, information regarding the rate of formation for such adducts (obtained either experimentally or by calculations) is extremely useful in grouping chemicals into mechanistic clusters. *In chemico* reactivity databases built with model nucleophiles (e.g., glutathione) are available for profiler development within the Toolbox. Currently, these are the only experimental data available to build chemical categories and these are incomplete.

It is self-evident that a compound cannot exert sensitisation-related reactivity in deeper layers of the epidermis unless it has penetrated the upper layers first (Basketter et al., 2007). Hence, bioavailability in skin sensitisation is often thought of in the context of dermal absorption. While considerable effort has been directed toward quantifying penetration of the stratum corneum of the skin (Guy, 2009), no accepted model currently exists. Dermal absorption is concentration-dependent; however, the exact relationship between concentration and absorbed percentage is not well understood (Buist et al., 2009).

An ancillary event in identifying protein-binding is metabolism and/or abiotic transformation (e.g. autoxidation) (Lepoittevin, 2006). *In vivo,* the keratinocyte is the primary site of metabolism in the skin (Smith & Hotchkiss, 2001). While *in silico* methods for identifying reactive metabo-

lites exist (Dimitrov et al., 2005; Patlewicz et al., 2007; Roberts et al., 2007), their current predictivity varies depending on the reaction being simulated.

Outcomes of *in vitro* assays vary with the type of cell used and the biomarker analysed. No single cell line or single biomarker is currently able to distinguish all *in vivo* sensitisers from *in vivo* non-sensitisers. Currently, the Keap1/Nrf2/ARE/EpRE cell signaling assay (Natsch & Emter, 2008; Emter et al., 2010) offer a straightforward means of measuring reactivity at the cellular level of organisation in a high throughput fashion. Genomic and proteomic studies also have the potential to reveal biomarkers (Hooyberghs et al., 2008; McKim et al., 2010).

While dendritic cell-based systems have been shown to distinguish a sensitiser from a non-sensitiser, outside of h-CLAT, current test results are somewhat limited in regard to the number of chemicals tested and the number of protein-binding reactions evaluated. Because they are quantitative, the h-CLAT CD86 EC150 and H-CLAT CD54 EC200 assays (Sakaguchi et al., 2009; Ashikaga et al. 2010) and the more recently developed IC20 endpoint of the CD34-DC assay (Hooyberghs et al., 2008; Lambrechts et al., 2010) have the potential to be *in vitro* endpoints which will aid in distinguishing strong, moderate, and weak sensitisers.

Proliferation of allergen-specific memory T-cells is a key event along the AOP, which is quantified with the LLNA. While sophisticated *in vitro* assay for this

key event are under development, it is unknown if they will provide information not captured in earlier events. However, there are some indications that sensitisers might induce a different repertoire and frequency of effector T cells, depending on their potency (Martin et al., 2010).

The AOP proposed in this case study provides a transparent, chemical and biological mechanistic-based framework for developing or refining chemical categories, as well as proposing and prioritising targeted *in vitro* and *in vivo* testing. However, the depth and breadth of available data currently does not allow for decisions associated with quantitative risk assessment.

Disclaimer

The views expressed in this paper are the sole responsibility of the authors and do not necessarily reflect the views of the OECD and its member countries.

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Alternatives Research in Japan

High-Throughput Assay Systems for Carcinogenicity, Immunotoxicity & Developmental Toxicity Developed by the METI/NEDO Project*

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Background & Objectives

Strategic R&D for the Safety of Chemicals in Japan

Approximately 5,000 high production volume (HPV) chemicals are now used by industries around the world, despite insufficient safety data. The Ministry of Economy, Trade and Industry (METI) in Japan is undertaking efforts to collect biodegradation, bioaccumulation and health/ecological effect data for such industrial chemicals. To accelerate HPV chemical safety data collection, and more generally to promote chemical risk analysis by industries, the New Energy and Industrial Technology Development Organization (NEDO), a research and development management organisation under the auspices of METI, initiated strategic research and the development of chemical risk analysis technologies in 2000. Several projects were started to develop such chemical risk analysis technologies. One of these projects is to develop the high-throughput (HTP) assay systems that use cultured cells and gene expression profile datasets in 28-day repeated dose oral toxicity studies.
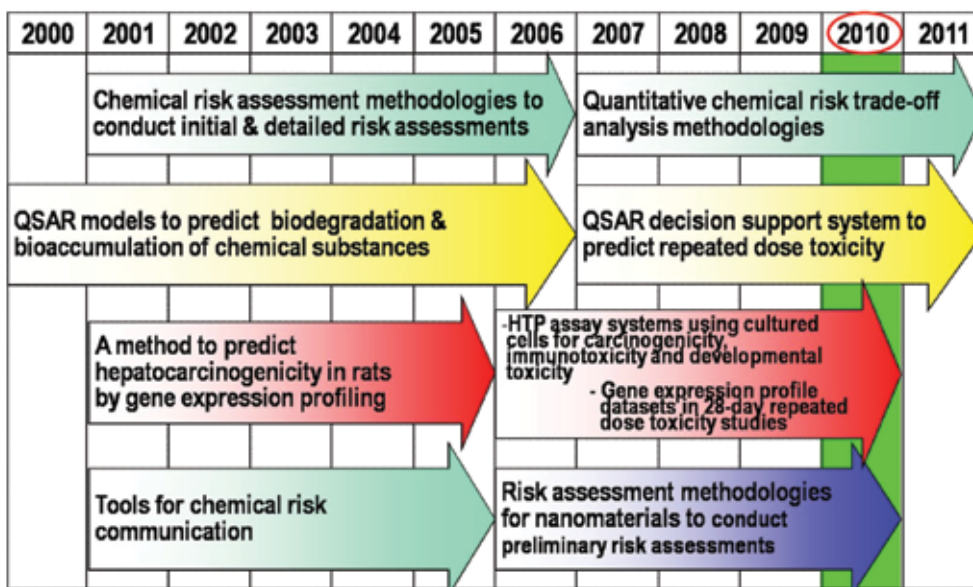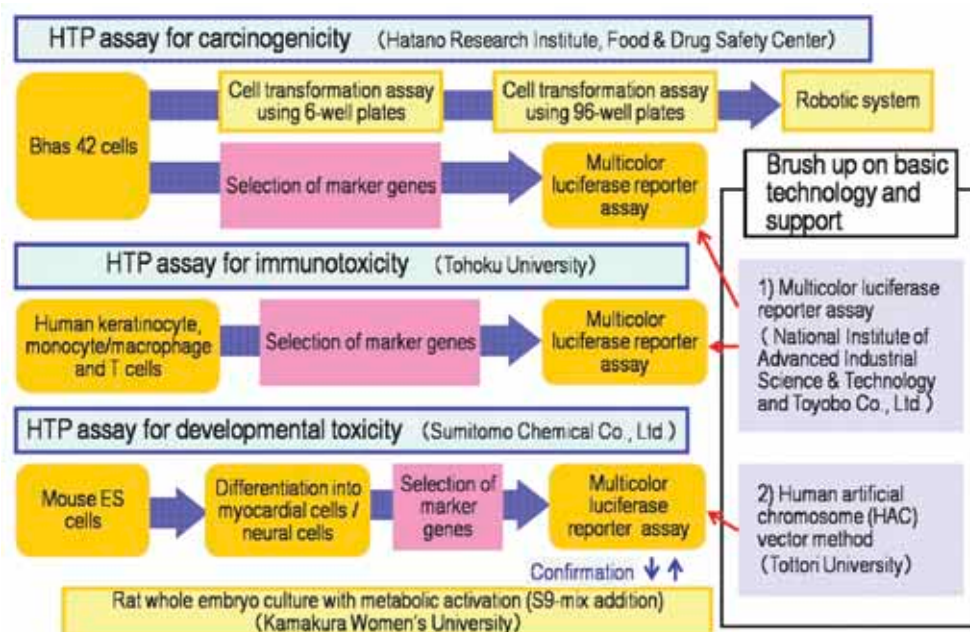
Figure 1. Strategic R&D of chemical risk analysis technologies in Japan.

Figure 2. R&D structure of NEDO project.

The first series the development of HTP *in vitro* assay systems was initiated by NEDO in 2006 and was finished in 2010 as a 5-year project. The second series of 5-year projects will be started in 2011 for the development of HTP assay systems to detect toxicities such as hepatoxicity, nephrotoxicity, etc. based on 28-day repeated dose oral toxicity tests (Figure 1). The objective of this presentation is to introduce novel HTP assay systems developed by NEDO project members in the 1st series (2006-2011) into practical use.

The R&D being conducted by NEDO (Figure 2) is aimed at establishing HTP assays of carcinogenicity (in partnership with the Hatano Research Institute, Food and Drug Safety Centre), immunotoxicity (Tohoku University), and developmental toxicity (Sumitomo Chemical Co. Ltd.). In addition to these toxicity research groups, technical support groups which have a high-tech multi-colour luciferase reporter assay [National Institute of Advanced Industrial Science and Technology (NIAIST) and Toyobo Co., Ltd.] and human artificial chromosome (HAC) vector method (Tottori University), were involved in this consortium.

HTP Assays for Carcinogenicity Using Bhas 42 Cell Transformation

Background

Although multi-stage processes are involved in carcinogenesis, the classical classification of carcinogens as initiators and promoters is practical for the screening of carcinogenic chemicals. Most initiators can be detected by various genotoxicity tests, the results of which are used for carcinogenicity prediction by regulatory authorities. In the case of tumour promoters, several methods have been proposed, but none have been routinely applied for regulatory purposes. Using cell transformation assays (CTA), BALB/c 3T3 cells and C3H10T1/2 cells can mimic the process of two-stage animal carcinogenesis. The formation of transformed foci is the consequence of the complex process of transforming cells to malignant state. Since these assays can detect both initiating and promoting activities, it is expected that they will be included as screening tools, but they have yet to be approved as routine screening methods because of the laborious and time-consuming procedures compared to routine genotoxicity assays.

In the NEDO project, a sensitive short-term CTA has been developed using Bhas 42 cells that were established from BALB/c 3T3 cells transfected with v-Ha-ras genes.
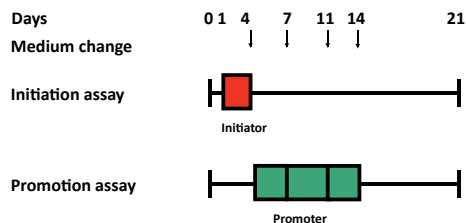

Figure 3. Bhas 42 CTA protocol for initiation assay and promotion assay. 6-well or 96-well plates are used. The cells for cell growth assay are fixed.

The Bhas 42 system can sensitively examine much more chemicals, including non-genotoxic carcinogens, in a short period (Figure 3).

Protocol of Bhas 42 CTA

Exposure schedules in Bhas42 CTA are illustrated in Figure 3. In the initiation assay, cells were treated with test chemicals in the growing phase for 3 days, then kept in cultures by changing culture medium. In the promotion assay, cells were treated with chemicals from the sub-confluent stage for 10 days. The cells for the transformation assay are fixed on day 21. The cells for the cell growth assays are fixed on day 7.

Advantages of Bhas 42 CTA

The Bhas 42 CTA has a number of advantages compared with conventional BALB/c 3T3 CTA. These include that: 1) Bhas 42 CTA is able to detect both initiators and promoters independently by slightly modified protocols; 2) Bhas 42 CTA is able to omit treatment with a tumour initiator in the promotion assay; 3) the culture period is shorten from 4-6 weeks to 2.5 to 3 weeks; 4) the number and size of culture vessels are possible to be decreased because of high transformation frequency; 5) Bhas 42 CTA is able to detect a considerable number of Ames-negative and Ames-discordant carcinogens by means of a promotion assay, confirming that Bhas 42 cells acted as initiated cells in CTA; 6) the ability of Bhas 42 CTA to predict the carcinogenicity of chemicals is superior or equivalent to genotoxicity assays; and 7) a new staining method using H_2O_2 treatment for objective measurement of living transformation foci has been developed to apply HTP system (Figure 5).

Thus, the Bhas 42 CTA is highly reliable to identify both genotoxic and non-genotoxic carcinogens, and it can be used as a
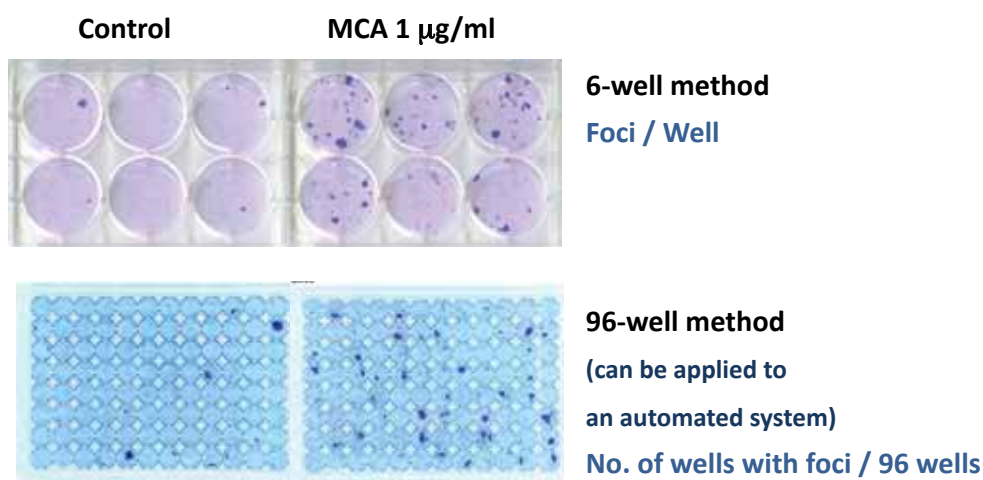

Figure 4. Data expression of transformation frequency in 6-well and 96-well methods.

Conventional method
Subjective
Time consuming for counting
Need fix & staining
Difficult to automate

New H$_2$O$_2$ method
Objective
Short measurement time by counter
No need fix & staining
Easy to automate

Figure 5. Advantages of Bhas 42 CTA using H2O2 method for HTP system.

powerful tool for predicting carcinogenic potential of chemicals (Figure 6). The accuracy of predicting chemical carcinogenicity would be improved by introducing Bhas 42 CTA into the battery of *in vitro* assays.

Through the electroporation or lipofection of these plasmids together with the G3PDH reporter plasmid, the cell lines shown in Table 1 have been established.

HTP Assays for Immunotoxicity

Selection of Immune-Related Marker Genes and Establishment of Reporter Cells

Based on DNA microarray analysis NEDO has conducted over the last 3 years, the following 7 immune-related genes were selected to evaluated the immunotoxicity of chemical: HMOX-1, IL-1b, IL-8, IFN-g, IL-10, and IL-22. They constructed plasmid vectors in which the expression of either the SLG or SLO luciferase gene was regulated by the promoter of each gene.
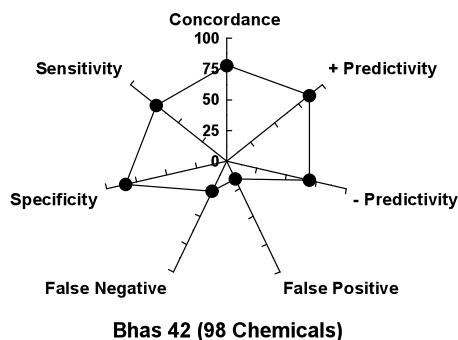

Bhas 42 (98 Chemicals)

Figure 6. Performance of Bhas 42 CTA tested with 98 chemicals.

Table 1. Established reporter cell lines for immunotoxicities.

Parent cells		Transfected genes			Cell lines
		SLR	SLG	SLO	
Jurkat	Single colour	G3PDH	-	-	#14D1
	Dual colour	G3PDH	IL4	-	#4A4
		G3PDH	-	IFNγ	#2B12
	Triple colour	G3PDH	IL4	IFNγ	#10C6
		G3PDH	IL2	IFNγ	#2B12、#2F12、#2H4、#2A12、#2A5、#7C2、#8D5
U937	Single colour	G3PDH			#UGA12、#9A6
	Triple colour	G3PDH	IL1β	IL8	#4D5, #6C12, #7A9
THP-1	Single colour	G3PDH	-	-	# TGC17

HaCaT	Single colour	TK	-	-	
	Dual colour	TK	HO-1	-	#HR38H6
U937	Single colour	TK	-	-	#UR2
	Dual colour	TK	HO-1	-	#UR2H4117
THP-1	Single colour	G3PDH	-	-	# TGC17
	Dual colour	G3PDH	IL1β		# TGC17bA16.19
	Dual colour	G3PDH		IL8	# TGC17EA01
	Triple colour	G3PDH	IL1β	IL8	# TGC17bA16EA26

Candidate Cell Lines for Immunotoxicity with Chemicals

NEDO combined the reporter cells (HMOX1, IL-8, IL-2 and IFN-g reporter cells) and established an evaluation system for many types of immunotoxicity with chemicals. Three distinct types of immunotoxicity were recognised: The first is T cell immunotoxicity related to immunostimulation, Th1/Th2 imbalance or immunosuppression. The second class is dendritic cell/macrophage immunotoxicity related to immunostimulation, Th1/Th2 imbalance, and sensitisation. The third class is keratinocyte immunotoxicity related to ROS production or redox imbalance.

Several papers have been reported that IL-8 production or mRNA expression by either monocyte-derived dendric cells (MoDCs) (Toebak, 2006), U937 cells (Python, 2007), or THP-1 cells (Nukada, 2008; Mitjans,2010; Arkusz, 2010) can provide a promising *in vitro* tool for discrimination between allergens and irritants. In the project, finally, a IL-8 reporter cell line, TGC17EA(=THP-G8) originated from THP-1 was listed as a good candidate for *in vitro* HTP assay of skin sensitisation.

In-House Data of In Vitro Sensitisation Test Using a IL-8 Reporter Cell Line: THP-G8

Up to now, many chemicals have been tested for allergenicity and irritancy using THP-G8 cells by the IL-8 Luc assay. The outcome of this assay showed very good performance with high specificity (90%), accuracy (86%) and sensitivity
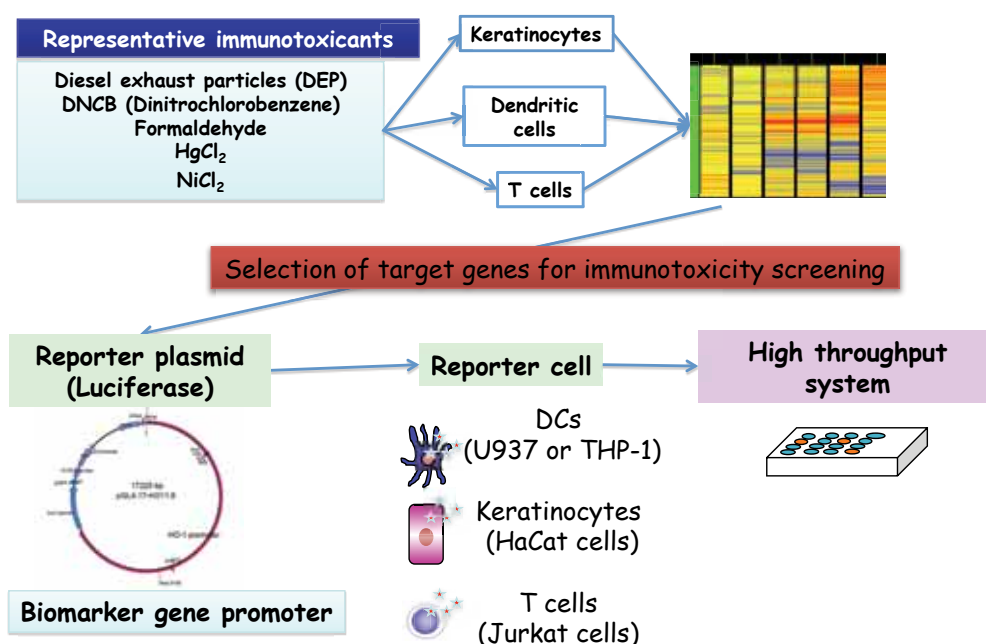
Figure 7. Development of HTP assay system for immunotoxicity.

(83%), and also low frequencies of false positives (9%) and false negatives (17%). These data were superior to those in the reports on h-CLAT assay (Ashikaga T. et al. Altern Lab Anim. 2010; 38, 275-84) and Keratinosens (Emter R. et.al. Toxicol Appl Pharmacol. 2010; 245, 281-90). The THP-G8 test system has various advantages, a short-term test period, simplicity of test protocol, etc., by using a multi-reporter assay.

HTP Assays for Developmental Toxicity

Objectives

A number of *in vitro* systems have been proposed as tests for developmental toxicity. However, it could be argued that these tests are unlikely to gain widespread acceptance and use. To establish convenient and accurate *in vitro* short-term assays for developmental toxicity, NEDO developed novel *in vitro* tests using mouse ES cell (Figure 8).

Selection of Marker Genes & Establishment of Transgenic ES Cells for Reporter Gene Assay

Firstly, suitable methods to differentiate mouse ES cells into cardiomyocytes and neurons were determined. Then gene expressions of ES cells during differentiation into cardiomyocytes and neurons were analysed, respectively. Finally, the following 13 cardiomyocyte-

related genes (Hand1, ADAM19, Cmyal, Pitx2, Smyd1, Pim2, Tbx20, My14, My17, Hbb-bh1, Hba-a1,Col1a2 and Hba-x) and 22 neural-related genes (Arx, Dcx, L1cam, Six3, Emx2, Reln, Ndn, Map2, Pax5, Basp1, Cpe, Ddr1, Nnat, Ptbp2, Marcks, Sfrp2, Sox11, Ttc3, Tubb2b, Ubqlnn2, Vim and Wnt1) were nominated as embryotoxicity markers by bioinformatics analyses. Identification of marker genes in ES cells was performed by treating them with embryotoxic and non-embryotoxic compounds. To generate of transgenic ES cell lines, selected marker genes were prioritised by gene function and expression profiles. Luciferase reporter vectors containing the promoter site of each marker gene upstream of the luciferase gene were constructed. Thus, a stable transgenic ES cells for detection of the

marker gene expression were established. Basic protocols for reporter gene assays using transgenic ES cells and investigation of efficacy were established using some standard compounds.

Progress

NEDO identified several genes whose gene expression was altered specifically by embryotoxicants during ES cell differentiation into cardiomyocytes and neural cells. These were selected as marker genes for detecting embryotoxicity. Several stable transgenic ES cells with the luciferase reporter gene were developed to detect the chemical dependent changes in the marker genes easily and conventionally. In order to establish appropriate basic operating protocol for an
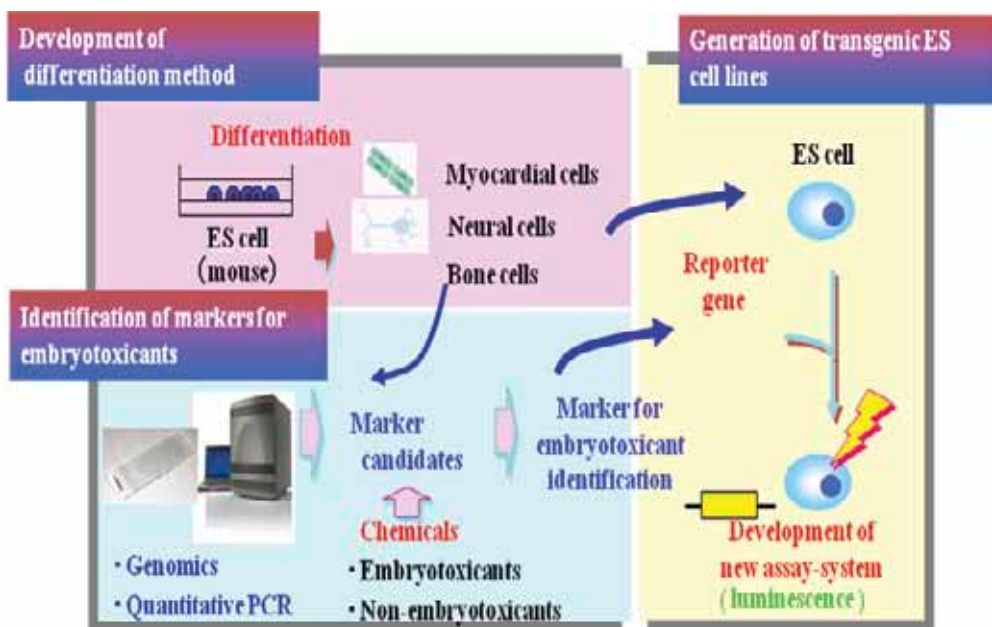

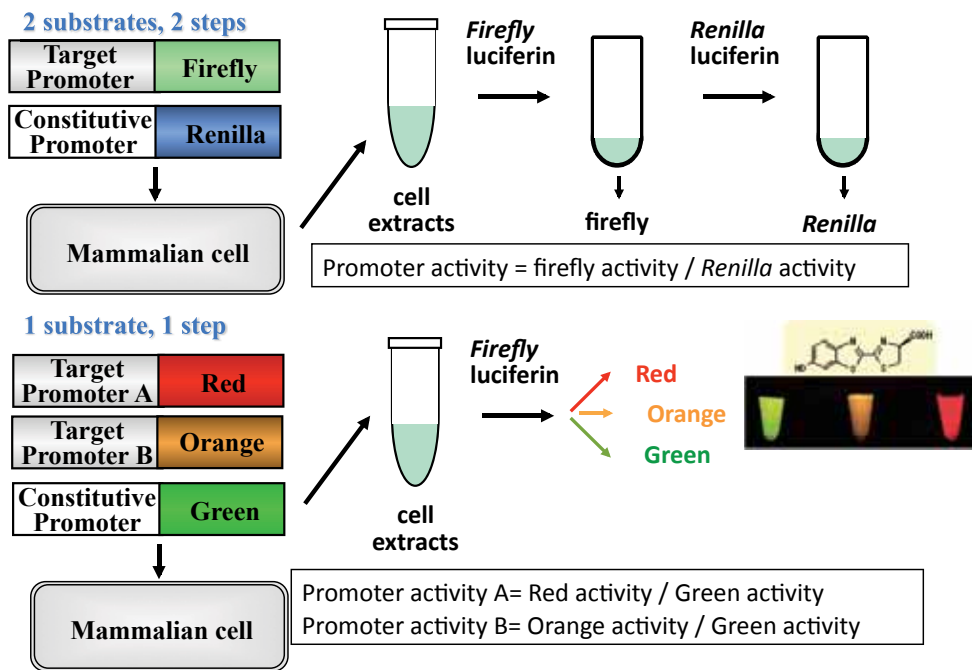Figure 8. Establishment of transgenic ES cell lines for embryotoxicity.

2 substrates, 2 steps

| Target Promoter | Firefly |
| Constitutive Promoter | Renilla |

↓

Mammalian cell

cell extracts → *Firefly* luciferin → firefly → *Renilla* luciferin → *Renilla*

Promoter activity = firefly activity / *Renilla* activity

1 substrate, 1 step

Target Promoter A	Red
Target Promoter B	Orange
Constitutive Promoter	Green

Mammalian cell

cell extracts → *Firefly* luciferin → Red / Orange / Green

Promoter activity A= Red activity / Green activity
Promoter activity B= Orange activity / Green activity

Figure 9. Schematics of the dual luciferase reporter assay system and the multicolour luciferase assay system.

in vitro short-term assay for prediction of embryotoxicity, NEDO optimised various conditions of the assay method. Next, several embryotoxic and non-embryotoxic chemicals were evaluated using NEDO's transgenic ES cells according to the basic protocol. NEDO has developed basic protocols for reporter gene assay using these transgenic ES cells and preliminary studies have been conducted using some standard chemicals in order to clarify the efficacy of NEDO's proposed tests. The outcome of these assays showed very good performance as compared with EST method.

Next Steps

The second series of 5-year projects under METI will be started in 2011 for the development of HTP assay system to detect toxicities such as hepatoxicity, nephrotoxicity, etc. based on 28-day repeated dose oral toxicity test. To promote the next 2nd series project, the main research center will move from Hatano Research Institute to Tottori University, which possesses a high-tech human artificial chromosome (HAC) vector method. Also, the multicolour luciferase reporter assay (NIAIST) will be applied for the development of HTP assays (Figure 9). In addition, JaCVAM will play an important role in this project.

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Case Study Approaches for Implementing the 2007 NRC Report

Toxicity Testing in the 21st Century: A Vision & A Strategy

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Background & Objectives

The 2007 NRC report has been extensively discussed in the toxicology and risk assessment communities (Andersen & Krewski, 2009; Krewski et al., 2009). The ongoing dialogue refined the vision and highlighted key issues about a shift to *in vitro* tests for assessing risks of chemicals (Andersen & Krewski, 2010). These issues were: (1) how will we define adversity from *in vitro* tests; (2) how will the *in vitro* test results be used to predict expected outcomes in animals and people who come in contact with the test compounds; (3) how will regulatory agencies set exposure standards for human populations based on *in vitro* test results; and (4) how will countries accustomed to the current whole-animal testing procedures move to another platform for testing and risk assessment? These questions squarely pose the core challenges that need attention in order to develop 21st century toxicology for both collecting toxicity testing information and interpreting the data for purposes of human health risk assessment. A key contribution to catalysing change will be case studies that show each component of this new paradigm in action and see how the new approaches address these four issues. This update describes progress at The Hamner Institutes for Health Sciences in designing case studies, developing generic test methods, bioinformatics, genomics and computational modelling tools for the case studies, and organising educational materials. A case study approach has also been endorsed by the Human Toxicology Project Consortium (*htpconsortium.org*), an effort coordinated through the Humane Society of the United States family of organisations.

Risk Assessments from *In Vitro* Test Results

An essay on the manner in which *in vitro* test results will provide measures of adversity (Boekelheide & Campion 2010) sparked a broader dialogue on a more explicit process to develop human exposure guidelines from an *in vitro* concentration deemed to cause an adverse cellular response (Boekelheide & Andersen, 2010; Bhattacharya et al., 2011). The likely process (Figure 1) has several steps involved in going from the *in vitro* adverse assay concentration to a regulatory standard. Chemical risk assessment would start by testing in a suite of 'validated ' *in vitro* assays and determining the pathway assay or assays that had responses at the lowest test concentration. Computational systems biology pathway (CSBP) models would utilise these dose-response curves and predict transitions from sub-threshold doses, to doses causing adaptive changes in the pathway function, on to those doses expected to have adverse consequences. These CSBP models would be developed for each pathway assay to understand the structure of the network in which the pathway is embedded and the dose-dependent activation of different portions of the signalling network. The integrated
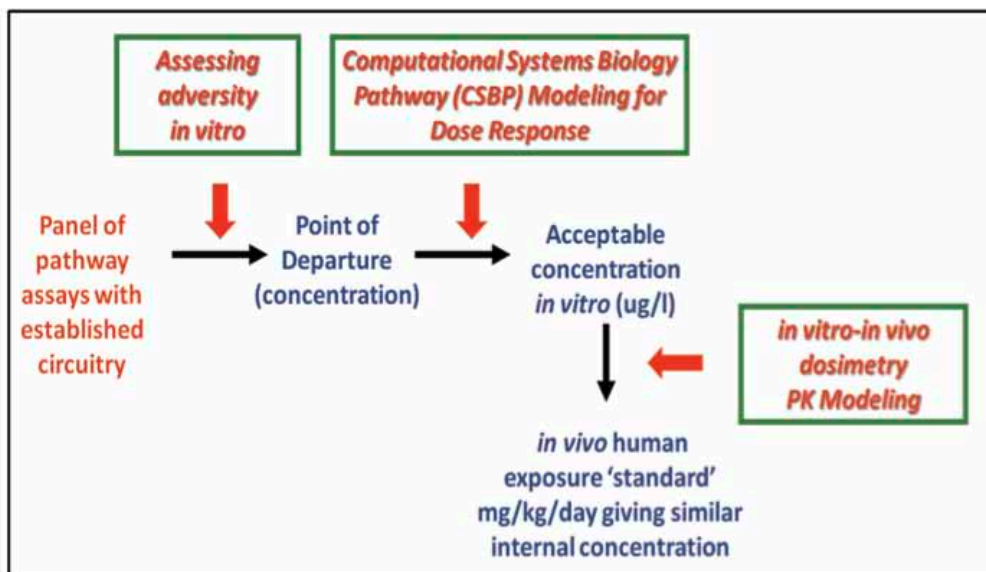

Figure 1. A Schematic showing likely steps in a toxicity pathway-based risk assessment. Results from the panel of 'validated' assays identify the pathway targets and generate a point of departure for the subsequent risk assessment as an *in vitro* concentration. Computational systems biology pathway (CSBP) modelling predicts the expected shape of the dose response at lower doses, leading to an acceptable concentration proposed for a human population. The acceptable concentration is then converted to an exposure standard through techniques of reverse dosimetry implemented by pharmacokinetic modelling. This step takes advantages of *in vitro-in vivo* extrapolation (IVIVE) and reverse dosimetry.

assay results together with CSBP models would provide the 'acceptable' *in vitro* concentration. Then, together with policy considerations, such as sensitive populations, response variability in a diverse population, etc., the *in vitro* adverse concentration would be adjusted to give an acceptable human plasma concentration. The last step in this process, estimating the *in vivo* human exposure expected to produce the *in vitro* concentration, would rely on technologies referred to as *in vitro-in vivo* extrapolation—IVIVE (Shiran et al., 2006; Gibson & Rostami-Hodjegan, 2007)—and reverse dosimetry (Clewell et al., 2008).

Case Study Approaches

A case study approach takes well-studied compounds that are known to affect specific pathways and runs them through a process equivalent to that shown in Figure 1. Candidates for these case studies are compounds that have been studied with conventional *in vivo* testing methods and whose mode-of-action has been well-characterised from both the *in vivo* test results and secondary mechanistic research. The mode-of-action framework activities over the past decade could serve as the basis for selecting one or more of the candidates (Sonich-Mullin et al., 2001; Boobis et al., 2006; Boobis et al., 2008; Julien et al., 2009). Case study approaches with prototype compounds require the availability of appropriate assays for specific cellular toxicity pathways and genomic/bioinformatic/computational tools to create CSBP models. For specific test compounds, IVIVE methods require

either chemical or chemical class specific approaches for kinetic modelling to describe expected pharmacokinetics and support reverse dosimetry.

The Hamner has developed a research proposal for a case-study approach to accelerating TT21C (*thehamner.org/institutes-centers/institute-for-chemical-safety-sciences/toxicity-testing-in-the-21st-century*). While still in the process of seeking funding, the programme has corralled sufficient resources to begin moving the case-study approach forward. The contributors to our current programmes are noted in the acknowledgments. Broadly, the Hamner efforts focus on both receptor-mediated toxicity pathways and pathways associated with chemical reactivity, such as DNA-damage and oxidative stress pathways (Simmons et al., 2009). Our research on the various pathways does not yet cover all aspects (Figure 1) for any single toxicity pathway; however, all the areas from the figure are under study with one prototype or another. The remainder of this report outlines, at a high level, activities in these various areas. The list of publications and expected publications for 2011 provides representative titles for the various research activities underway.

Progress Update: Assays

Human Cells In Culture

For studies with the DNA-damage related pathways, we have developed high-content imaging and flow cytometry methods to visualise and quantify markers of DNA-

damage (H2AX binding to DNA and micronuclei formation) and activation of the p53 response pathway, including p-53, p-p-53, p21 expression and cell cycle arrest (Lahav et al., 2004; Bryce et al., 2008; van Attikum & Gasser, 2009). Currently, the work uses a human osteosarcoma cell line, HT-1080. This cell line has functioning native p53 (Benchimol et al., 1982). The goal in this project is to have a suite of assays at the cellular level that link DNA-damage, pathway responses to DNA-damage, and mutation. Responses across a range of cellular responses (Figure 2) are necessary as the data are taken forward to the CSBP model for DNA-damage. The lead PI for the work is Dr. Rebecca Clewell, working with a post-doctoral fellow, Dr. Bin Su.

Organotypic Tissue Culture

Useful toxicity pathway assays require fidelity of the responses in the *in vitro* systems with *in vivo*, or at least with intact human cells. Most of the current q-HTS tests are not conducted in intact human cells. We are developing organotypic liver culture systems that will contain multiple cell types and maintain function for weeks at a time to provide an improved *in vitro* test system. This project, led by Dr. Ed LeCluyse, is evaluating several commercial organotypic liver models using two prototype compounds—acetaminophen and a PPAR-a agonist—GW7647. Once the systems are optimised, we will use
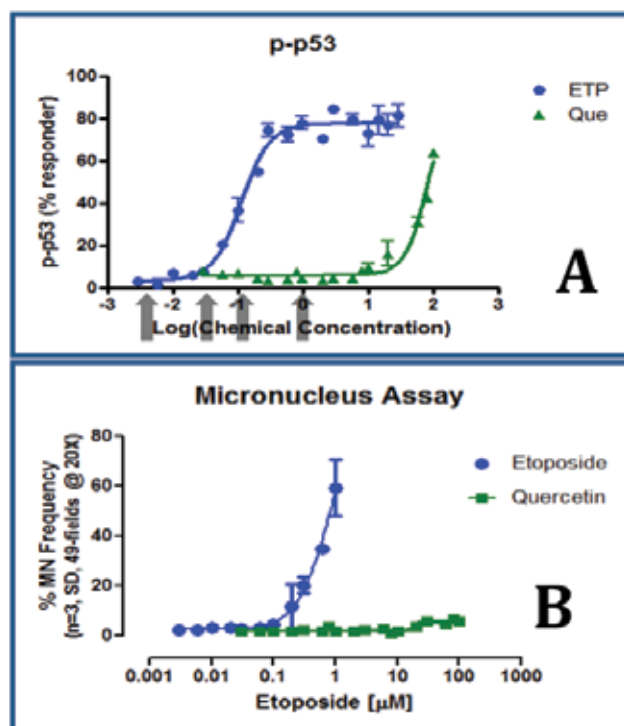

Figure 2. From assay results to networks. DNA-damage pathway research effort uses flow cytometry and high content assays to asses cell responses (phospo-p53 in Panel A; micronuclei formation in Panel B) with several compounds expected to have specific mechanisms of DNA-damage in HT-1080 cells, an adherent human osteosarcoma cell line. These two compounds, etoposide (a topoisomerase inhibitor) and quercitin (expected to be active through oxidative stress pathways) have divergent dose-response curves. The DNA-damage dose response curves are then used to identify treatment concentrations (grey arrows for proposed studied with ETP) that would be used to assess the pathway circuitry over exposure concentration and time in order to extract information on dose-dependencies in network structure and develop a CSBP model of the network.

them to support multi-day testing in order to see if these systems provide a good linkage between *in vitro* cellular assays and *in vivo* whole animal responses. These organotypic liver cultures should also prove valuable in assessing metabolic pathways for test compounds since the culture could also function as a bioreactor not simply as an assay system. While not suitable for high-throughput, the advantages of multi-day dosing, metabolite formation, and culturing with multiple cell-types could make this system a test bed for showing the advantages of more mechanistic, medium-throughput *in vitro* assays.

Progress Update: CSBP Modelling

Circuitry

The TT21C vision is that there will be a suite of accepted standardised assays that are predictive of the activity of chemicals toward specific toxicity pathways. Each assay will be validated using positive controls, such as 17-ß-estradiol for an estrogen pathway assay. In addition, especially in the step that links assay results to dose-response assessment, it will be necessary to develop the 'wiring diagramme' of the network with an understanding of the manner in which the pathway is embedded in an overall response network and how the dynamics of the pathway arises from its topology. Validation—in the sense of fit-for-purpose—is the understanding the manner in which the network circuitry controls different levels of response (sub-threshold, adaptive and adverse) for the

pathway assay system.

Network Inference & Dose Response

Over the past two years, the Hamner programme in this area has focused on PPAR-α, with special attention to examining the time and dose dependence of this network in primary hepatocytes from humans and rats. The studies dose and time dependencies of gene expression and transcription factor binding after treatment with a PPAR-α activating ligand (GW 7647). The study design follows that used to establish the network for the cannabinoid receptor (Bromberg et al., 2008). An abstract of our PPAR-α work appeared earlier this year (Woods et al., 2010). We have also been developing bioinformatic tools to automate aspects of network inference from genomic and DNA-binding data sets. The structure of these networks would be a set of nodes connected in a logical pattern leading to a sequential dose-dependent network. The resulting circuit structure controls gene expression, in a manner similar to what has been called a 'developmental network' (Alon, 2007). These networks are expected to have several nodes and feed-forward loops that give rise to different levels of activation as concentration increases (Figure 3). These network can lead to both monotonic and non-monotonic behaviours of families of gene products over time, as seen in uterine gene expression due to estrogen treatment in ovariectomised rodents (Kwekel et al., 2005). The multiple signalling nodes are also compatible with layered, cascade-models for responses to estrogen, androgen, and other endogenous

hormones (Landers & Spelsberg, 1992).

With the DNA-damage pathway, our next steps will move from the development of the assay for DNA-damage markers, such as micronuclei and mutation, on to collection of gene expression and p53-D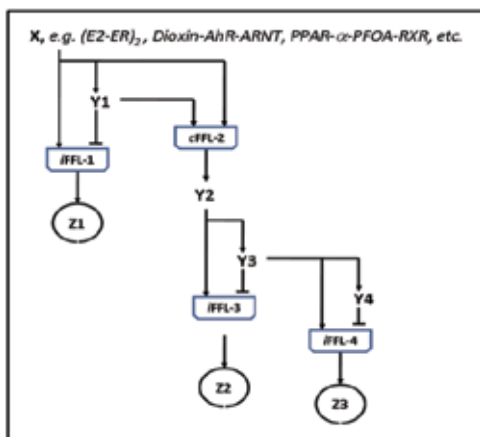NA binding (Figure 2), to infer network structure from bioinformatics methods based on gene expression and transcription factor binding (Bromberg et al., 2008; Shen et al., 2011). In general, these steps—assay development, network inference for the toxicity pathway assay, and the completion of the CSBP model to assess dose-dependencies in network activation—would all be collected before the assay was deemed 'validated' and ready for use.

CSBP Models

A variety of modeling tools are now available and have become widely used in the biomedical engineering community (Aldridge et al., 2006). Our primary efforts with CSBP models are currently focused on p53-mediated DNA-damage and repair networks. High-dose responses of p53-meaidted DNA-damage pathways have been examined through iterative experimentation and pathway modelling from the biomedical community for the past 10 years (Lahav et al., 2004; Batchelor et al., 2008; Batchelor et al., 2009; Loewer et al., 2010). The Center for Dose-Response Modeling at The Hamner is refining published DNA-damage models for describing perturbations of the p53-signalling network and will apply these models to a series of chemicals with different mechanisms by which they affect DNA—etoposide (a topoisomerae inhibitor), quercetin (a pro-oxidant natural product), and methylmethane sulfonate (a DNA-alkylating agent).

Our intention is to develop several case studies that include network inference and

Figure 3. A schematic for a 'developmental network'. Networks of this kind have been described by Alon (Alon, 2007), and implemented in Hamner Computational Systems Biology and Dose Response Modelling Workshops. The network is controlled by several incoherent and coherent feed forward loops (iFFL and cFFL) with nodes to either activate (arrows) or repress (blunt lines) gene expression. The goal is to understand the circuitry for various toxicity pathways at this level of detail in order to describe exposures that are without appreciable effects, those causing activation of early portions of the pathway, and those producing full activation. These dose dependencies are expected to overlay with the areas of subthreshold, adaptive and adverse perturbations as outlined in the NRC report. In this network, proteins designated Z1, Z2, and Z3 would have non-monotonic dose response curves; Y1, Y2, Y3 and Y4 would have monotonically increasing, but sequentially delayed, responses.

CSBP models for both receptor-mediated and stress-mediated pathways. These tools are part of the process of assay development, determination of fitness of the assays for purpose, and using deep understanding of network biology to consider dose-dependent transitions in network activation. Hamner staff have developed courses in computational cell biology and dose-response (*thehamner. org/about-the-hamner/education-training/ dose-response-modeling*) and presented a continuing education course (CEC) on computational cell biology at the 2011 US Society of Toxicology meeting in Washington, DC. The bare-bone model for a developmental network (Figure 3) has been used in courses at the Karolinska Institute and Michigan State University. The code can be obtained by contacting the authors.

Progress Update: *In Vitro-In Vivo* Extrapolation

Specific Compounds

The validated pathway assays provide response measures for test compounds (e.g., an EC50, a Benchmark concentration, an adverse response concentration, or an interaction threshold identified by the CSBP dose-response model for the toxicity pathway of concern). *In vitro* to *in vivo* extrapolation (IVIVE) then assists in estimating the environmental exposures to a chemical that could produce target tissue exposures in humans equivalent to those associated with effects in an *in vitro* toxicity test. Through a combination of quantitative structure-property relationship (QSPR)

modelling, physiologically-based pharmacokinetic (PBPK) modelling, and collection of *in vitro* data on metabolism, transport, binding, etc., IVIVE provides estimates of the likelihood of harmful effects from expected environmental exposures (Clewell et al., 2008).

High-Throughput Applications

In collaboration with US EPA's ToxCast programme, Hamner staff developed generic dosimetry models by measuring metabolic clearance in primary hepatocytes, plasma protein binding and by estimating renal filtration from protein binding. These data were input to a population-based *in vitro*-to-*in vivo* extrapolation programme (SIMCYP™) for estimating the human oral equivalent dose necessary to produce a steady-state *in vivo* concentration equivalent to *in vitro* AC_{50} (concentration at 50% of maximum activity) and LEC (lowest effective concentration) values from the ToxCast data. These calculated daily exposures are compared to human exposure levels to assist in deciding which compounds are likely to be more problematic (i.e., produce blood levels overlapping with measures of bio-activity from the *in vitro* studies). The first paper using this approach, a reverse dosimetry methodology referred to as reverse toxicokinetics, evaluated 35-compounds from the Phase I chemicals examined by ToxCast (Rotroff et al., 2010). A second paper in review has applied these reverse dosimetry tools to virtually the entire ToxCast Phase I group of compounds (Wetmore et al., papers in review). In other research, Hamner staff

are developing IVIVE models for specific compounds, such as phthalates, parabens, carbaryl and arsenic (Choi et al., 2011).

Training

Hamner staff from our Center for Human Health Assessment (CHHA), led by Dr. Harvey Clewell, held week-long courses, one in 2010 and the second in 2011, that included lectures on using PBPK modelling to conduct IVIV extrapolation for cell-based toxicity testing (*thehamner.org/about-the-hamner/education-training/pbpk-modeling/#2011*). The course material is available on the website. The Hamner group also led a continuing education course (CEC) on IVIVE at the 2011 SOT meeting. A Hamner-led proposal for a CEC on early life dosimetry and vulnerable populations has been accepted for the 2012 SOT meeting.

Conclusions

Progress in developing case studies requires careful selection of prototype compounds and prototype pathways. In addition, contributions are necessary from a trans-disciplinary staff with diverse skills—assay design, genomics/bioinformatics, computational modelling, pharmacokinetics and human health risk assessment. Our programmes are moving forward with resources that are targeted to specific technical areas and slowly expanding to develop several case studies that span all the key technical areas required for success with any individual pathway assay. We are furthest along with case studies for the p53-DNA damage and the PPAR-a pathways. Work with these prototypes provides activities covering each aspect of risk assessment based on *in vitro* test results (Figure 1).

The case study approach offers several advantages. First, it does not worry excessively over all challenges required to make a wholesale change in toxicity testing, focusing instead on the process by which *in vitro* toxicity information will/could be used for setting regulatory standards in specific instances. Second, it proposes learning by doing. Many key issues in use of the information will become apparent by moving ahead with the process. We can look tour own history and suggest that only a couple of successes with case studies will make the entire process go along much more easily. The two authors of this report were among a small group who brought PBPK modelling forward for use in risk assessment in the 1980s. It is an area that now has contributed widely to human health risk assessment. Based on our experience, the majority of challenges required to implement PBPK modelling to a diverse set of compounds were clearly defined with the application to the first two compounds—styrene and methylene chloride (Ramsey & Andersen, 1984; Andersen et al., 1987). History is likely to repeat itself with toxicity testing in the 21st century. After completing the first two or three pathway case studies, most of the issues will become clear and expansion of the testing to other pathways will be greatly accelerated.

Hamner Presentations in 2011

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- Woods CG, Yarborough KM, Pluta LJ, et al. Constructing a PPARa-Mediated Transcriptional Network in Primary Human and Rat Hepatocytes. The Toxicologist 2011; 78.

Key Pending 2011 Publications

- LeCLuyse EL, Witek RP, Andersen ME, et al. Organotypic liver culture models: Meeting the future challenges in toxicity testing. Under review.
- Wetmore BA, Wambaugh JF, Stephen S, et al. Integration of dosimetry, human exposure and high-throughput screening data in the toxicity assessment of environmental chemicals, Toxicol. Sci., Submitted.
- Yoon M, Campbell J, Andersen ME, et al. *In vitro* to *in vivo* extrapolation of cell-based toxicity assay results. Crit. Rev. Toxicol. In-house review.

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Safety Assessment at Unilever Applying 21st Century Toxicology

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Background & Objectives

In 2004 Unilever initiated a long-term research and capability development programme designed to evaluate and implement a new conceptual approach for assuring consumer safety without animal testing (Fentem et al., 2004; Westmoreland et al., 2010). The strategic approach and ambition of this internal programme align closely with the vision and strategy for 'Toxicity Testing in the 21st Century' published by the US National Research Council (NRC) in 2007 (Krewski et al., 2010).

Unilever's approach for safety assessment is risk-based, meaning that all available data on a new ingredient (including predicted levels of consumer exposure during product use) are used to assess the level of risk posed by its proposed consumer use. The scientific challenge we are investigating is how, in the future, novel *in vitro* data and *in silico* predictions may be used within this risk-based framework. Specifically we are investigating:

1. Risk-based approaches to assuring safety in the area of skin allergy (underpinned by a systems biology approach to understanding the mechanistic basis of skin sensitisation) (Maxwell et al., 2008; Wexwell & MacKay, 2008).
2. A case study (DNA damage-induced carcinogenicity) to evaluate the potential application of a toxicity pathways-based approach within a risk assessment context for repeat dose toxicity (Bhattacharya et al., 2011). This case study (in collaboration with the Hamner Institutes for Health Sciences, US) is designed to investigate the applicability of the principles outlined in the US NRC's report.

There is a significant scientific challenge to understand how safety may be assured for complex toxicological endpoints using data derived from a toxicity pathways-based approach that is rooted in mechanistic understanding of the underlying biology. An equally important challenge is how such toxicity pathways-based approaches could ultimately be incorporated into regulatory frameworks for the safety assessment of chemicals and products.

Safety Risk Assessment in Unilever

Unilever's evidence-based approach is built on the core principles of 'safety by design and in execution' and 'safety is non-negotiable' across its foods, home care and personal care product portfolio. All key safety concerns are identified early in the innovation process and managed pro-actively. Safety risks are assessed and managed along the R&D—Supply Chain—Consumer Use continuum (Figure 1). Safety decisions are risk-based (made on an assessment of 'acceptable risk'), taking into account the probable exposure / use of the products by consumers as well as any potential hazards and likely exposures associated with the raw materials (ingredients), product formulation and manufacturing process.

When evaluating the safety of new technologies and products, Unilever applies existing scientific knowledge and, where necessary, generates new hazard and exposure data to complete safety risk assessments. These assessments underpin decisions on risk acceptability and any necessary risk management measures (e.g. information provided to consumers via product labels). Unilever's scientific evidence and risk assessments are included in safety dossiers submitted to regulatory authorities globally.

Scientists at Unilever's Safety & Environmental Assurance Centre (SEAC), part of the company's global R&D organisation, provide safety and risk / impact assessment expertise and guidance for the company worldwide. They are responsible for: (a) the scientific quality and transparency of assessments of consumer, occupational and environmental safety risks and environmental impacts for Unilever's key technologies, products and processes; (b) ensuring Unilever has access to the capability needed for safety risk and environmental impact assessments; and (c) developing and applying robust approaches for risk and impact assessments based on up-to-date science and in accordance with global scientific standards. The core technical expertise areas of SEAC are in toxicology and ecotoxicology, environmental science and sustainability, chemistry, microbiology and process safety, with focus on their application for hazard characterisation, exposure and risk assessments, and life cycle assessments. Many of SEAC's scientists are recognised experts in food and chemical safety, alternative approaches to animal testing, and environmental sustainability. They work collaboratively with research partners across the globe to develop new scientific capability in safety risk and environmental impact assessments.
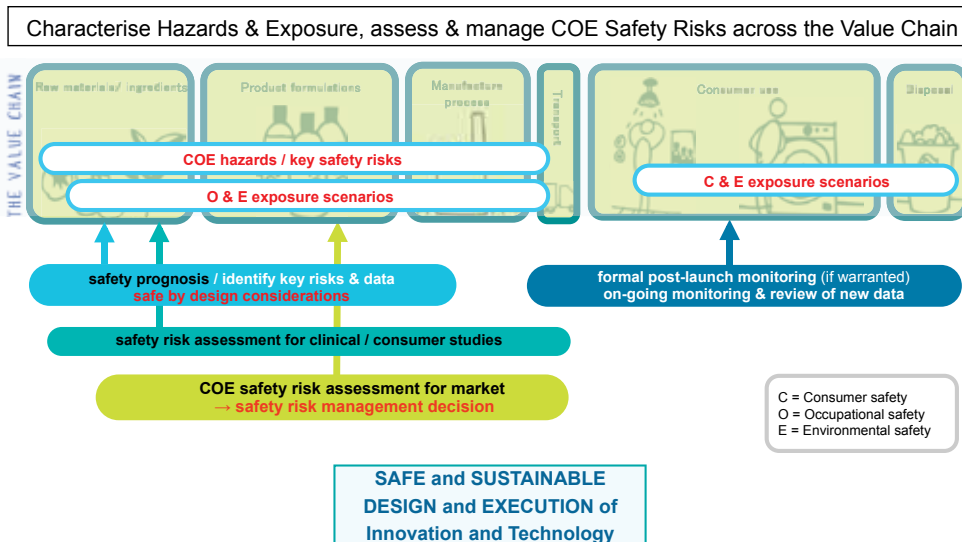
Figure 1. Safety risk assessment in Unilever; integrated approach to assessing and managing risks.

These are exciting and challenging times for toxicology as well as for other fundamental biological sciences. There is a clear modernisation agenda within toxicology, driven to some extent by the US NRC report. Advances in science and technology have the potential to transform the human health risk assessment paradigm and future chemicals risk management approaches and regulations (enabling a step change in the use of non-animal approaches and data). The strategic direction is to move away from some of the traditional hazard characterisation approaches (typically using laboratory animals) to far greater application of new cellular and molecular methodologies, and to better understanding of human biological pathways and networks and how these are affected by exposure to chemicals. Ultimately, such approaches will improve the scientific quality and

robustness of our toxicological (human health) risk assessments and risk management decisions. SEAC scientists are very active in looking to understand how best to apply the most up-to-date scientific knowledge and approaches in this area, to avoid the need to generate any new animal data within the context of Unilever's integrated, risk-based framework for safety risk assessment.

Non-Animal Approaches for Consumer Safety Risk Assessment

Over the past 20 years, SEAC scientists have established a strong scientific track record in developing and applying non-animal approaches for assessing consumer safety. This includes, for example, implementation of: (a) *in vitro* tests for skin corrosion, skin irritation,

phototoxicity and eye irritation, focussed principally on hazard identification; (b) *in vitro* methods for skin penetration, and investigative metabolism and kinetics studies; (c) exposure-based approaches such as the toxicological threshold of concern (TTC) and assessments of the risks associated with inhalation of particulates; and (d) chemistry-based approaches such as structure-activity relationship (SAR), read-across and substantial equivalence assessments.

Since the 1980s, Unilever's commitment to eliminating animal testing for the safety assessment of its products has been underpinned by a scientific research programme to develop alternatives to animal tests. From 2004 an additional €3 million a year has been invested to evaluate a new conceptual framework for an integrated, risk-based approach to the application of non-animal approaches for assuring consumer safety. Current research is focused in two main areas: (1) the development and evaluation of new risk assessment approaches for assuring consumer safety in the areas of skin allergy; and (2) a case study (DNA damage-induced carcinogenicity) to understand the potential application of a pathways-based approach to toxicity within a risk assessment context for repeat dose toxicity (in collaboration with the Hamner Institutes for Health Sciences, North Carolina, US; *thehamner.org*).

The scientific and technical challenges associated with assuring consumer safety without any animal testing are enormous and it is clear that no single research group or company will achieve these goals alone. Therefore, Unilever works in partnership with many external groups in addition to having its own in-house research programme within SEAC's laboratories in Sharnbrook (UK) and Bangalore (India). These partnerships include sponsorship of research with academic institutions, investigating new approaches with contract research organisations, initiating bespoke research with biotechnology companies, and consultancies with key experts.

Unilever is also actively involved in EU-funded research projects; participates in cross-industry collaborative research, e.g., that conducted under the auspices of the European Cosmetics Association (COLIPA; *colipa.com*) and the European Partnership for Alternative Approaches to Animal Testing (EPAA; *epaa.eu.com*); and has ongoing involvement with other scientific initiatives on non-animal approaches to risk assessment, e.g. with the UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs; *nc3rs.org.uk*) and the US-based 'Human Toxicology Project' Consortium (*htpconsortium.org*), which is looking to follow up on the NRC 'TT21C' strategy and vision from 2007 (Stephens, 2010).

In 2004 Unilever published a suggested future direction for replacing animal testing for human health risk assessment via the integrated application of new technologies and new non-animal models (Stephens, 2010; Fentem & Westmoreland, 2011). The question posed was "how

can consumer safety risk assessments benefit from applying new and emerging technologies being used in medical and biological research?" Subsequently, in partnership with other leading research teams, Unilever has invested in developing new capability in "omics" (genomics, proteomics, metabolomics), informatics (computational and mathematical approaches covering chemistry and biology), analytical, and bioengineering (tissue constructs) technologies and tools (e.g. for integrating, analysing, visualising and interpreting diverse types of non-animal data) (Westmoreland et al., 2010; Maxwell et al., 2008; Maxwell & MacKay, 2008; Fentem & Westmoreland, 2007).

The EU Scientific Committee on Consumer Safety (SCCS) published an updated opinion on the status of alternative test methods in human health safety assessment of cosmetic ingredients in the EU in 2010 (SCCS, 2010). The conclusion of the report was that the "majority of the existing alternative methods [are] only suitable for hazard identification of cosmetic ingredients and do not give information on potency. Thus, a full human health risk assessment cannot be performed." A comprehensive review, by experts selected by the European Commission, on the current scientific status of alternative methods for cosmetics testing was published recently (Adler et al., 2011). This summarises research being undertaken aimed at replacing animal tests for the key human health endpoints of: (a) repeat dose toxicity (general systemic effects, including adverse effects on target organs

such as liver, kidney, heart, etc.), (b) carcinogenicity, (c) reproductive toxicity, and (d) skin sensitisation, plus research into non-animal methods for generating toxicokinetic data. In many cases, research programmes for these endpoints are still at an early stage (e.g., SEURAT-1, the cluster of projects on repeat dose toxicity being funded jointly by the European Commission and COLIPA; *seurat-1.eu*).

The SCCS (2010) opinion and the outcome of the European Commission's expert review (Adler et al., 2011) indicate that in future greater priority needs to be given to developing non-animal approaches which provide biological and chemical dose-response data which can be integrated into consumer exposure and safety risk assessments. The strategic direction articulated in the US NRC report, and efforts to develop new approaches around 'adverse outcome pathways' (Schultz, 2010), offer a promising way forward in this context.

Toxicology in the 21st Century

Unilever's research over the past 8 years into new approaches to skin allergy risk assessment without animal testing aligns well with the NRC's 'TT21C' concept (MacKay, 2010). Mathematical modelling was applied to improve understanding of the mechanistic basis (biological pathways) of skin sensitisation. The insights gained then informed new research objectives. Currently, new metrics of skin sensitisation hazard are being evaluated. It is envisaged that these will enable *in vitro* data to be used for skin

allergy risk assessment rather than just for identification of the sensitisation potential (i.e. hazard) of chemicals.

Unilever's capability development projects in this area now include an evaluation of the feasibility of a TT21C pathways-based approach to safety risk assessment, in collaboration with the Hamner Institutes for Health Sciences (case study: chemical-induced DNA damage; toxicity pathways associated with DNA damage and repair). Such case studies will provide the evidence base on which to progress mapping the human toxome as proposed recently by Hartung & McBride (2011).

Like skin allergy, cancer also represents an extremely important endpoint in the safety assessment of consumer products. Past strategies for the safety assessment of DNA-reactive chemicals have relied heavily on results from *in vitro* tests (genetic toxicology tests) being confirmed by 'definitive' animal studies (genetic toxicology and carcinogenicity tests). We believe that a new non-animal strategy can be developed that is more informative and ensures safety to the consumer.

In vitro-only genetic toxicology assay strategies have a high irrelevant positive rate (i.e., positive results will be obtained for chemicals that are not carcinogenic, and many common food-based phytochemicals, e.g., flavonoids, can be rejected erroneously if *in vitro* regulatory tests are employed alone. This is because of the inherent nature of the current assays. On-going research is focussed on identifying approaches to increase the

specificity of currently available *in vitro* genetic toxicology tests. Since these tests are used purely in a hazard identification mode, the label of 'genotoxicity' indicated by the current tests necessitates the rejection of that chemical if no follow-up testing is conducted. However, greater evidence and wider acceptance of the existence of thresholds for genotoxic events, determined in *in vitro* systems, is emerging (Carmichael et al., 2009). This may provide a way forward for the risk assessment of new chemicals. High-throughput methodologies are being used, such as automated micronucleus scoring, to provide the data necessary for low-dose determinations of thresholds, in standard and newly engineered cell lines.

The characterisation of a material as 'low-dose thresholded' will require adequate understanding of the molecular mechanism of action of carcinogens, and dose-response information regarding changes in the toxicity pathways involved. 'Omics technologies offer opportunities to produce data needed to generate this understanding. Successes with transcriptomics and metabolomics have shown discrimination between chemicals with probable thresholded characteristics, based on mechanistic understandings (e.g., the activation of DNA repair pathways, changes in the cell cycle and oxidative/metabolic stress). It is hoped that novel insights that are currently being generated will be capable of informing a new risk-based approach. Several other new technologies are also being investigated, to increase our understanding of the complex interactions that occur

in biological systems in response to carcinogenic chemicals. For example, infra-red micro-spectroscopy is proving to be valuable in mapping and understanding the transformation of Syrian Hamster Embryo cells in culture, in response to chemical carcinogen exposure.

The challenge ahead is to integrate the dose-response data obtained *in vitro* from the toxicity pathways associated with chemical-induced DNA damage with information on consumer exposure and modelling of subsequent systemic exposure (e.g., using physiologically based kinetic modelling), to enable risk assessments to be performed for new chemicals in consumer products under the conditions of use. The application of systems biology approaches to anchor these *in vitro* measurements to relevant biomarkers and pathology (adverse outcome) pathways will be core to this approach.

Next Steps

The results from Unilever's research programmes in skin allergy and chemical-induced DNA damage confirm our belief that an essential element for success is the involvement of multidisciplinary teams able to draw upon new developments in multiple science and technology fields. For example, our progress in the area of skin allergy is a result of successful teamwork between chemists, immunologists, cell biologists, mathematical modellers and bioinformaticians within Unilever, and across our network of external partners. SEAC scientists are now looking to help

build effective bridges between EU and US research initiatives, to engage Chinese scientists in this research, and to ensure full alignment with TT21C concepts that recognise the importance of exposure (dose-response) data and the application of data derived from new technologies within a risk-based safety decision-making framework. In March 2011, the opportunities that the TT21C vision and strategy offer for a new approach to assuring safety without the generation of animal data were discussed at a meeting organised by Unilever at its R&D laboratory in Shanghai (China). Some of the authors of the US NRC report met with leading Chinese scientists and regulators, and scientists from Unilever to start to plan how China participates with TT21C research and education initiatives (*tt21c. org*).

While the key scientific challenge is to actively pursue new research objectives clearly aligned to understanding biological pathways and outcomes critical for human health risk assessments (here a clear roadmap would help increased international scientific collaboration and focus), an equally important challenge for the future is how, if successful, such toxicity pathways-based approaches could ultimately be incorporated into regulatory frameworks for the safety assessment of chemicals and products. Traditionally, *in vitro* hazard identification tests (e.g. for skin irritation) have been validated as 1 for 1 replacements for an existing animal test, according to OECD, ECVAM and ICCVAM guidelines. However, it is likely that a pathways-based approach will involve a

'toolbox' of non-animal methods, none of which by themselves will be a replacement for a current animal test. Thus, tests based on understanding toxicity pathways for use in safety risk assessments will need to be evaluated for regulatory use by a different approach. US EPA scientists have taken the initiative in responding to this challenge, and are leading discussions on how best to validate high throughput pathway-based assays that are being evaluated under the Tox21 programme.

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3.4 Breakout Group Reports

Three breakout groups (BOGs) were established for a focused discussion of the scientific state-of-the-art and of knowledge gaps and priorities for future EU research funding.

In the first breakout group, general building blocks for a pathway-based paradigm were discussed, while the other two groups examined reproductive toxicity and sensitisation as case studies. All three BOGs were provided with a series of tailored thought-starter questions and the following general background information:

3.4.1 Background Information

A key outcome of the AXLR8-1 workshop in 2010 was an endorsement by the AXLR8 Scientific Panel of a 'top-down' research strategy in Europe based on the transformative vision of 'toxicity testing in the 21st century'. Scientific authorities agree that by developing a robust understanding of 'adverse outcome pathways'[1], i.e., the sequence of key events between a molecular initiating event and an adverse toxicological outcome at the whole organism level, it should be possible to shift future risk assessments toward avoidance of significant perturbations of normal cellular pathways/ networks by using a suite of *in vitro* assays to detect these perturbations. This requires exploration of the relationship between cellular pathways/networks and 'modes-of-action'[2], dose-response modelling organised around computational systems biology models of the 'circuitry' underlying each pathway, and extrapolations based on pharmacokinetic models to predict tissue concentrations under specific exposure conditions.

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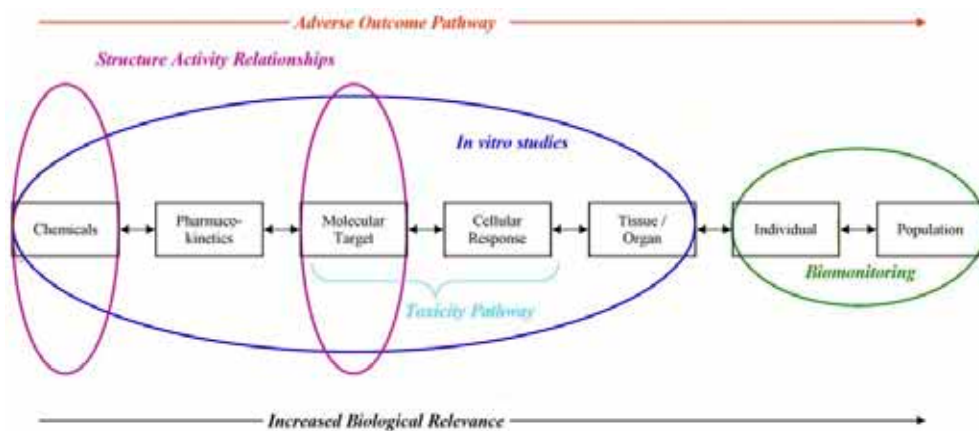
Figure 1. An illustration of the mechanistic pathway from a molecular initiating event through toxic outcome at the whole organism or population level (adapted from EPA, 2011[3]).

The 2010 AXLR8 Progress Report identified the following as priority areas for near-term research:

- Definition, identification and categorisation of key biological pathways in human toxicology
- Use of a systems biology approach to integrate toxicity pathway responses into a mode-of-action framework based on dose/concentration-response
- Characterisation of dose/concentration-dependent transitions in pathways and integration of responses across pathways
- Refinement of physiologically-based pharmacokinetic (PBPK) and multi-scale modelling and other bioinformatics tools to interpret the substantial amount of new data that will be produced under a pathway concept
- Proof-of-concept demonstration studies using known toxicity pathways and clinical symptoms/biomarkers to illustrate the pathway approach in practice.

The AXLR8 Scientific Panel further noted that EU funding schemes should emphasise 'value added' collaborations among established research teams in key areas (e.g., the US Tox21 initiative) to create synergies without duplication. Targeted multidisciplinary partnerships should also be encouraged, given that a solution for more predictive and animal-free safety assessment needs the mobilisation of the best scientists in their fields, many of whom would not traditionally apply their work to toxicology.

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[3] US EPA. Use of New Computational and Molecular Tools. FIFRA Scientific Advisory Panel Consultation, 24-26 May 2011. Washington, DC: EPA.

3.4.2. Break-Out Group 1: Cross-Cutting Topics

The goal of BOG1 was to discuss the building blocks of a 21st century paradigm in the context of existing European and international research presented during the AXLR8-2 workshop.

Participants

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Thought-Starter Questions

1. What are the critical 'building blocks' and key science issues of a pathway-oriented research strategy?
2. What are the core research questions behind each of these points?
   a. What tools and technologies (e.g., databases, AOP/MoA libraries, assays, etc.) are necessary to support such a research strategy?
3. To what extent are EU-funded projects exploring these questions already?
   a. Where are the key gaps?
   b. Where are there opportunities for international collaboration and synergy?
4. What should be the initial priorities for a pathway-oriented EU research programme?
   a. How might such a programme look in comparison to conventional models (e.g., division between 'health' and 'environment', size of consortia, international collaboration, etc.)?

Discussion Summary

• There was a general view among BOG1 participants that a shift in toxicology toward a pathway- and systems biology-based paradigm is needed to address substantial unmet needs under the current paradigm—including a backlog of insufficiently studied substances and mixtures, the high cost and inefficiency of *in vivo* test

methods, and the generally poor understanding of mode-of-action.

- The overarching vision articulated by the group is that of 'protection of human health through better safety assessment'. This approach is considered to hold promise also for the broader field of biomedical research vis-à-vis elucidation of human-relevant disease pathways, and should therefore not be seen as relevant only to the field of toxicology.

- A higher level of understanding of modes-of-action and their relationship to traditional endpoints is required in order to design novel test methods and strategies that are fit-for-purpose. Knowledge of the relationship between chemical structures and pathway perturbations feeds back into the innovation cycle supporting the development of 'greener' chemistries. The potential economic benefits of such innovation were also stressed by several participants.

- Participants discussed the relative advantages of the EU's traditional endpoint-oriented approach to alternative test method development compared to the more generalised and data/hypothesis-driven approach being followed in the US. Recognising several health/endpoint areas as near-term priorities from an EU legislative perspective (i.e., carcinogenicity, reproductive and developmental toxicity, immunotoxicity and sensitisation, and specific target organ toxicity), the group explored options for integrating key 'building blocks' (e.g., kinetic and multi-scale modelling, systems biology and computational chemistry, etc.) in a horizontal, cross-cutting manner to maximise alignment between EU and other regional research programmes and, by extension, the potential for future collaborations and synergies.

- A move away from organ-specific toxicity descriptors (i.e., liver or kidney work packages) is foreseen as mode-of-action knowledge and the associated ontology continue to develop. A consequence of this will be the emergence of a description of toxicological hazard in terms of key intermediate effects/events that are common across many modes-of-action, as apposed to a description of toxicity based solely on a specific apical adverse outcome.

- Interpretation of *in vitro* data in order to distinguish 'adaptive' from 'adverse' responses in human beings and populations will require a specialised framework rooted in a robust understanding of human systems biology. In the near-term, such a framework will be needed for interpretation of the vast quantities of data being generated, identification of knowledge gaps, development of hypotheses and establishment of future research directions.

- Approaches to validation of the emerging methods were briefly discussed. Use of available human material (e.g., oncology bio-banks comprised of tissues with well described pathologies; patient data including time-dependent profiles and metabolomics, exposure histories, etc.) was recommended. It was noted that conventional approaches to formal validation are cumbersome and not well suited to the evaluation of integrated pathway assays and modelling tools.

- Two distinct funding models were suggested for future EU research in this area: the large-scale 'research cluster' used in the 'SEURAT-1' joint research initiative, and the creation of a centralised and highly networked 'EU Centre for Molecular Toxicology' where scientists from different disciplines could work at one location toward the same well-defined aim, and through which research and training could be co-ordinated in a focused way. A majority of participants favoured the first option.
- These objectives could also be pursued through Augmentation of the EU's research and technology infrastructure (e.g., high-throughput and high-content analysis, next-generation sequencing, data- and knowledge-bases, imaging, bio-banking, genetically modified cell lines, stem cells, bioengineering of artificial tissues and organs) was identified as a priority if European research is to keep pace with developments in other regions (*pathogenomics.bham.ac.uk/hts*). The concept of a cross-cutting 'infrastructure cluster' was suggested as a core component of future funding efforts in this area. The establishment of a new collaborative research institute was also suggested.
- Greater flexibility to more readily permit mid-stream course corrections, e.g., recruitment or expulsion of partners, was identified by several participants as a desirable characteristic for future EU funding programmes.
- Several participants commented on the perceived 'artificial separation' between EU Framework Programme thematic funding areas (i.e., health, environment, food), noting the potential for knowledge of environmental systems to inform human health assessments and vice-versa. For instance, exposure and biomonitoring are generally examined and funded under the environment theme, yet data from such studies are critical for a risk-based approach to human safety assessment. The growing desire on the part of regulatory authorities for more unified and integrated assessment criteria was also noted.

3.4.3. Break-Out Group 2: Reproductive Toxicology

BOG2 members discussed the current incentives and challenges in Europe for improving the established methods in developmental and reproductive toxicology and made recommendations on a scientific framework that has to be established to implement innovative toxicity testing in this area of toxicology, identified the tools that are required and made a key recommendations on improving the scientific basis, on funding requirements and on making an impact on the public perception of the safety testing paradigm.

Participants

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Thought-Starter Questions

1. Which are the essential steps of pre- and postnatal development that have to be covered by an innovative non-animal testing approach?
2. Which of these endpoints are covered by new methods developed in recent EU (ReProTect, ESNATS) and US projects (ToxCast, Virtual Embryo).
3. Have additional promising tests covering the essential endpoints been developed and which institutions provided funding?
4. Are there any essential endpoints for which innovative non-animal methods have not yet been developed and what are the most promising approaches to cover these endpoints as well?
5. Which of the innovative methods are covering 'toxicity pathways' and is it essential that 'adverse outcome pathways' are covered?
6. Are there any promising approaches to integrate the new and innovative methods that will allow covering the whole reproductive cycle or parts thereof?
7. From the perspective of funding, which sections of the reproductive cycle should get then highest priority, developmental or reproductive toxicity testing?

8. How should studies in lower species be included, *C. elegans*, *Drosophila* & zebra fish?
9. Should DNT (developmental neurotoxicity) studies be given high priority?

Discussion Summary

Current scientific, public and political expectations in Europe

The current EU cosmetics directive points to an elimination of animal testing for ingredients of cosmetic products in 2013. The current research trajectory will not be capable of meeting this deadline with adequate non-animal alternative assays. In particular, a focus on improving developmental and reproductive toxicity testing is necessary to achieve this goal in the near future. In addition, alternative approaches are needed in developmental and reproductive toxicity since these studies are particularly animal-intensive study types. More effective and efficient methods will be a benefit to both the regulated industry as well as the regulatory authorities, who will be able to better prioritise their resources. Consumer protection will be enhanced by developing innovative toxicity tests that can be applied to greater numbers of chemicals that cover the key biological pathways related to induction of reproductive dysfunction and developmental anomalies.

The way forward in developmental and reproductive toxicity testing

- We must build on novel experimental evidence, e.g., as provided by the EU FP6/7 projects ReproTect, Chem*Screen* and by the US ToxCast and Tox21 initiatives, and incorporate new and innovative approaches to probe relevant biology
- Predicting safety for humans is the ultimate concern; therefore, we need to identify disease pathways in human development and reproduction and examine existing scientific information for evidence of chemical effects on these pathways in order to discover key biological pathways.
- Research should focus on the use of human-derived molecules and cell systems and on developing assays and models for key pathways where gaps are observed
- Phenotypic gaps should be identified in the fields of fertility (i.e., oogenesis, implantation and placentation in females; spermatogenesis in males; and postnatal diseases in general, such as neurological, immune and cardiovascular diseaes, diabetes and obesity)
- Promising biological pathways should be coverer, e.g.:
    - Relevant developmental signalling pathways (e.g., WNT; stem cell studies show promise as a surrogate for this currently)
    - Transcription factor profiling (e.g., cardiac defects)
    - Biochemical trapping of protein targets (e.g., Thalidomide example)

- Deep-sequencing of birth defect patients and generation of iPCS as research tools
- An adequate supporting Infrastructure must be established, i.e., bioinformatics, chemical reference (and quality control library), central data repositories that utilise common ontologies; high-throughput robotic facilities
- Potential case study: Provide a context relevant Integrated Testing Strategy that includes all relevant levels of biological organisation and ADME/TK in an efficient and effective manner using systems biology as the central organising principle. Robust proof-of-concept studies should be conducted to demonstrate applicability and feasibility, recognising the differing regulatory requirements between data-rich and-data poor chemicals.

Tool development is essential

- Production of human iPSC cells of different genotypes and lineages (shared with other EU projects such as regenerative medicine and SEURAT-1) will provide novel tools in developmental and reproductive toxicology in addition to mouse and human embryonic stem cells (ESC)
- Organotypic cultures (bioreactors) should be established for study of emergent properties of tissues of reproductive organs, e.g., oogenesis and spermatogenesis
- Development and application of use of non-mammalian model organisms should be established, e.g., zebrafish; the current state of science suggests utility of zebrafish for both hazard identification and mechanism-of-action studies
- Metabolism (ADME) needs to be part of the integrated, innovative testing strategy including reverse-toxicokinetics and description of kinetic situation of the model systems
- Multi-scale models should be developed that integrate lower-level molecular assays through cellular function to apical manifestations of adverse reproductive outcomes; this includes modular approaches to various elements of the reproductive cycle that can ultimately be linked and integrated for endpoint prediction
- Integrated testing strategies should be developed that provide context relevant information for determination of chemical safety (e.g., drug-design food-use pesticide, commodity chemicals, etc.).

Key Recommendations

Scientific challenge in developmental and reproductive toxicology:

- Characterising impacts on key regulatory pathways using simple cellular assays, more complicated cellular systems, and model organisms in an integrated fashion
- Prioritisation scheme for ranking chemicals according to their *in vivo* adverse effects on

the various endpoints of developmental and reproductive toxicity.

Funding aspects:

- A long-term (5-10 year) developmental funding process is needed, as e.g., the SEURAT-1 project for repeated dose toxicity
- Joint public-private partnerships between the private sector NGOs and international governments are recommended, building on the positive experience from the SEURAT-1 project
- An integrated cluster of projects covering essential pathways is recommended.

Building public confidence in a new safety evaluation paradigm to:

- Prove that the new approach provides better protection for humans and/or the general public than the current approach
- Demonstrate that the transparency of the new approach in contrast to the current 'black box' concept
- Ensure that uncertainties are clearly understood.

Supplemental Background Information

Specific principles of reproductive toxicology

Mammalian reproduction is characterised by a rather complex reproductive cycle, the essential steps of which are depicted in Figure 2. Reproductive toxicology covers a wide spectrum of toxic effects at all stages of the reproductive cycle, starting with female and male fertility, prenatal and postnatal development, and culminating in late manifestations that can only be detected in the next generation. Thus, in contrast to other fields of toxicology, reversible and irreversible effects of exposure to toxicants may occur, not only in individuals that were exposed, but also in their offspring.

Risk assessment is particularly challenging in reproductive toxicology, due to the complexity and unusually long timeframe/duration of the reproductive cycle, which covers at least two generations. To assess the potential of chemicals to interfere with reproduction, animal-based test methods must cover these essential steps (Figure 2):

a) growth and maturation of sperm and oocyte;
b) fertilisation, e.g., fusion of oocyte and sperm, resulting in a complete, diploid set of chromosomes;
c) normal cleavage divisions, implantation, intrauterine development, birth, and postnatal development throughout the period of lactation; and
d) normal development of the offspring to fertile adult animals, which are able to
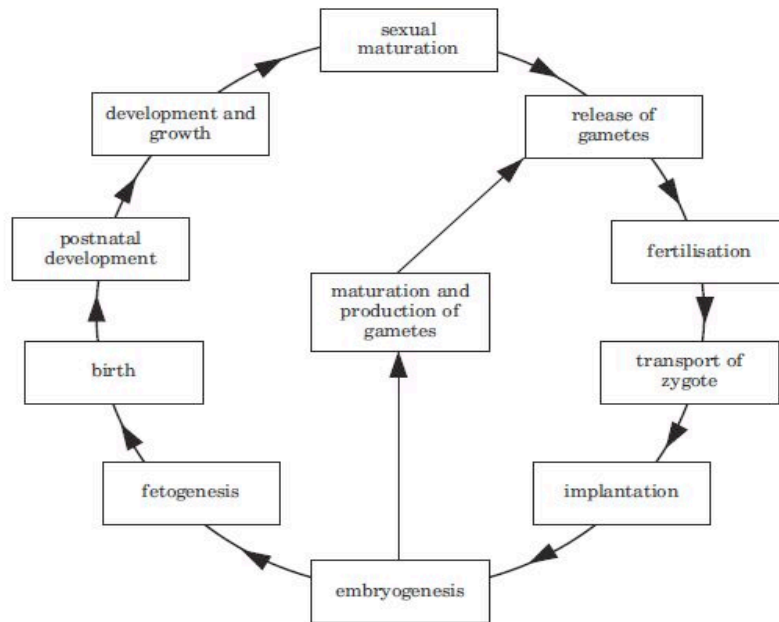
Figure 2. Specific stages of the mammalian reproductive cycle.

produce a second generation.

The complexity of the reproductive system and the vast number of tissue targets for the induction of malformations or postnatal effects, provide the rationale for the toxicity testing of chemicals in highly standardised and internationally harmonised animal tests, which are summarised in Table 1.

Drugs are tested according to International Conference on Harmonisation (ICH) test guidelines (7), which require a teratogenicity (Segment 2) study and prenatal (Segment 1) and postnatal (Segment 3) studies. Table 1 shows that industrial chemicals and certain chemical products must be tested according to EU and Organisation for Economic Co-operation and Development (OECD) test guidelines; in addition to the teratogenicity/developmental toxicity study, one-generation or two generation studies and even a developmental neurotoxicity study have to be performed in some cases to detect perinatal and postnatal effects. The implementation of the US High Production Volume (HPV) Challenge Program and the new EU REACH legislation are expected to result in a dramatic increase in the number of toxicological studies that rely on animals.

Table 1. International test guidelines in reproductive toxicology.

Test compounds	Test guideline
Drugs (ICH)[a]	Segment 1: Combined fertility and embryotoxicity study Segment 2: Teratology study Segment 3: Prenatal and postnatal study
Industrial and agrochemicals (OECD)[b]	Teratology study (TG 414) One-generation study (TG 415) Two-generation study (TG 416) Reproduction and development screening (TG 421) Combined repeated dose and developmental screening (TG 422) Developmental neurotoxicity study (TG 426) Uterotrophic assay, oestrogenic properties (TG 440) Hershberger assay, androgenic properties (draft TG) Human oestrogen receptor-α transcriptional activation assay (draft TG) Extended one-generation reproductive toxictity study (EOGRTS; draft TG)

[a]ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) test guidelines for drugs (7); [b]OECD Guidelines for the testing of chemicals (8, 34–39).

EU and US projects aimed at improving reproductive toxicity testing

The European Commission Directorate General for Research & Innovation has within the Health theme of FP6 and FP7 funded a series of projects in the programme 'Alternative Testing Strategies - Replacing, reducing and refining use of animals in research'. Within this programme, two large-scale Integrated Projects focused in particular on reproductive toxicology: the ReProTect (FP6) and ESNATS (FP7) projects. In ReProTect a series of non-animal methods were developed covering several important endpoints of the reproductive cycle. In a prevalidation (feasibility) study several of these tests showed a quite promising predictive value. In the ESNATS project the use of embryonic stem cells in toxicity testing is investigated and some promising results have emerged.

Table 2: Summary of results from the ReProTect feasibility/prevalidation study

Independently in the US, the Environmental Protection Agency has launched two projects focusing on specific aspects of reproductive toxicology: ToxCast and the Virtual Embryo project. In the session 'Case Study 1: Reproductive Toxicity' of the AXLR8-2 workshop, presentations of the EU ReProTect and ESNATS IPs and of the US ToxCast and Virtual Embryo projects will give an overview on progress achieved so far.

3.4.4 Break-Out Group 3: Skin Sensitisation

The aim of BOG3 was to: 1) evaluate whether the strategy used for safety testing of skin sensitisers using alternative approaches has been successful and the extent to which it has addressed already the points arising from the AXLR8 2010 workshop; 2) identify the areas were further work is needed for adequate prédiction of risks for chemically-induced skin sensitisation in humans; and 3) evaluate whether the experience gained from the research for alternatives to test skin sensitisers can be extrapolated to other applications and other 'nearby' areas of safety testing, e.g., respiratory sensitisation.

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Thought-Starter Questions

1. To what extent does the bullet-point list above capture the critical 'building blocks' of a pathway-oriented strategy for skin sensitisation toxicity testing? Are there any key science issues that have not been captured that are necessary elements to consider?
2. Which tools and technologies (e.g., databases, AOP/MoA libraries, assays, etc.) have been used behind each of these points? What tools need to be developed further?
3. To what extent are EU-funded projects exploring these questions already? Where are the key gaps? Where are the opportunities for international collaboration and synergy?
4. Which other aspects of sensitisation need to be further developed and covered by alternative animal free approaches e.g., respiratory sensitisation, individual sensitivity?

Discussion Summary

- There was a general view among BOG3 participants that progress in the field of skin sensitisation testing over the last 10 years has been substantial. Several research groups have unraveled key mechanisms related to chemically-induced skin sensitisation, which is a complex process, which involves the chemical reactivity of compounds with the surrounding extracellular matrix of the skin, as well as reactivity with specific cell types. Structure-activity approaches have defined key characteristics and an *in silico-in vitro* strategy is being developed to identify sensitising chemicals. The initial cellular events at the level of dendritic cells and keratinocytes are now largely understood. Advanced technologies such as genomics and proteomics have been introduced and have contributed to the elucidation of the sensitisation phase in keratinocytes and dendritic cells. This has enabled the development of a testing strategy based on mechanistic understanding of the crucial steps leading to skin sensitisation by low-molecular-weight chemicals.
- The current working strategy is largely in line with the pathway-based concept of '21st century' toxicology. The process was successful in unraveling basic immunological processes for skin sensitisation and yielding a test battery that covers the key essential pathways except for T-cell responses. BOG3 recommends further work to build on the success in the skin area and to apply a similar approach towards a testing strategy for respiratory sensitisation.

Challenges for the future:

- The BOG emphasises that a conceptual framework needs to be built on integration of already available building blocks of assays and computational knowledge. This integrating framework should not be developed empirically but should be based on mechanistic insights. New experimental data should be generated that permit validation of an integrated *in silico* simulation.
- Better consumer exposure scenarios are needed as an input into the testing framework. Human exposure pathways and dose patterns are not yet well defined. Information on the dose range of interest and the likelihood of continuous or repeated dosing should permit simulation of real-life exposures. These scenarios are needed to obtain useful results from the *in silico/in vitro* testing strategy, thereby addressing thresholds of adverse effects based on mechanistic insights.
- Realistic scenarios should include cost-effective models for topical application, and should also take into account that exposure to a sensitising chemical occurs typically in combination with other chemicals. Testing strategies should include the testing of formulations. Interaction with other ingredients such as irritants or impaired sensitivity due to damaged or diseased skin should be considered.
- The BOG identified in particular the lack of predictive clinical data as a drawback

for further progress. Better tools than the currently used patch tests are needed. Quantitative biomarkers in humans related to mode-of-action leading to adversity should be identified. The BOG recommended development of joint research programme to link critical steps and biomarkers in human disease models with molecular events in *in vitro* systems. This is expected to improve the predictivity of the *in vitro/in silico* models. Knowledge in the following areas remains limited: understanding metabolism in human skin, transport of sensitising chemicals through human skin, identification of critical proteins for complete antigen formation, understanding the T-cell response which is largely driving the adverse outcome pathway, and understanding individual differences among patients in response to the same sensitising molecules.

- Data and knowledge management is a cornerstone for future progress in this area. Prior to the initiation of future research programmes, existing knowledge obtained in innovative networks should be inventoried and introduced as building blocks into an integrated framework. Results and expertise of European projects such as Sens-it-iv and OSIRIS, and the research initiated and led by Colipa and its member companies, should be made widely available. Research networks have been built worldwideand the knowledge obtained should not be lost.

- A common ontology should be developed and knowledge management tools should be introduced to optimise capacity-building and dissemination of knowledge.

- The BOG recommends strongly that step-wise development of the framework should include also a proof-of-concept phase, which in an iterative way should lead to further improvement and refinement of the concept.

Supplemental Background Information

Different background documents summarise the state-of-the-art for testing skin sensitisers using alternative methods (OECD 2011; Adler et al., 2011). The process of chemical-induced skin sensitisation is fairly well understood (Kimber et al., 2010). The following steps are identified as key processes: an initiation phase that requires penetration of the chemical through the epidermis; binding of the chemical to skin proteins; epithelial inflammation; binding of the hapten-protein complex to dendritic cells (DC); maturation and migration of DCs; contact of DCs with naïve T cells; production and proliferation of memory-T cells. The inflammatory responses on the skin occur only after an elicitation phase, which requires a second contact of the chemical with the skin, penetration through the epidermis, and binding to skin proteins, activation of DCs, release of inflammatory cytokines by memory T cells, and recruitment of other inflammatory cells from the blood which migrate to the skin to induce local inflammatory reactions in the skin. Currently *in vitro* tests are available for many of these endpoints but their validity has not been formally validated and will not be available for risk assessment for 2013.

Combined set of tests will be needed for final evaluation. It is widely recognised that alternatives to animal testing cannot be accomplished with a single approach, but rather will require the integration of results obtained from different *in vitro* and *in silico* methods (Basketter & Kimber 2009; Roberts & Patlewicz 2010). The physicochemical diversity determines the domains of applicability of the available *in vitro* tests. An integrated testing strategy adapted to the diverse chemical categories is required. Questions to consider:

- Which elements are in place / which steps are further needed and are still missing?
- Are the endpoints under study specific enough for sensitisers, how do they relate to other biological processes such as oxidative stress responses?
- What is needed to reduce uncertainties for skin sensitisation testing? How to proceed?

To predict risk we need to have information on the dose at which chemicals exert the adverse biological effect

☐ Potency information enables the establishment of safe levels for human exposure to chemicals that cannot be regarded as not having any skin sensitising potential. An important question is whether there is a threshold below which no adverse effects are to be expected. With respect to the skin, dose-response relationships and no-effect levels were found for both intradermal and topical induction, as well as for intradermal and topical elicitation of allergenic responses in epidemiological, clinical, and animal studies (Arts et al., 2006). It was also shown that repeated exposure to low doses of contact sensitisers may increase the sensitising potency (Paramasivan et al., 2009). With the introduction of the murine Local Lymph Node Assay (LLNA) and the EC3 value (the effective concentration of test substance needed to induce a stimulation index of three), chemicals could potentially be placed into potency groups (e.g., extreme/strong, moderate, weak, and non-sensitisers (Basketter 2007). Is potency ranking possible using *in vitro* assays or computational models? Assays on peptide reactivity, QSARs and cellular systems are attempting potency classification of skin sensitisers (Gerberick et al., 2008; Van Och et al., 2005; McKim et al., 2010; Lambrechts et al., 2010). To date, experience has shown that using a combination of in vitro assays with in silico models might predict hazard classification. However challenges still exists for risk assessment decision-making. International efforts of research leaning on a shift of paradigm and relying on human cells as well as on adverse outcomes pathways are still needed. Another difficulty is the availability of reliable, preferentially human, *in vivo* data for potency ranking. Other drawbacks may be the limited solubility of the compounds for *in vitro* testing and the lack of oxidation and metabolism in the alternative assays. Knowledge of the dose at the target cells is another important issue. Relating *in vitro* and *in vivo* expo-

sure doses is not straightforward. Although we are able to compute phase partition behaviour for small molecules in for instance oil/water, this becomes a far more challenging issue in the body where membrane and cellular structures are critical. It is key to the understanding of the effective dose at the site of interaction.

Chemical sensitization is not limited to skin sensitisation

Skin sensitisation resulting in allergic contact dermatitis represents the most common manifestation of immunotoxicity in humans, and many hundreds of chemicals have been implicated as skin sensitisers. Fewer chemicals have been shown to cause sensitisation of the respiratory tract and asthma, but the issue is no less important because hazard identification remains a significant challenge, and occupational asthma can be fatal (Kimber et al., 2011). Biological pathways for respiratory allergy are different from those of skin allergies and involve activation of T helper (Th) 2 cells (Lambrecht 2010). Some of the skin sensitisers may also be lung sensitisers.

Needs for a systems biology approach to understand the unknowns of sensitisation

Although we partially understand the mechanisms by which chemicals induce and elicit sensitisation in the skin, we have ample understanding of parameters that influence sensitivity. Clinical studies have shown that that age, gender and genotype may influence the prevalence of allergic diseases (Omenaas et al., 2008; Blomeke 2009). Evidence is accumulating that prenatal exposure to some chemicals may interfere with sensitisation later in life, and while epigenetic pathways have been suggested, these remain to be elucidated (North 2011). This information is useful for individual risk assessments beyond the general assessments performed today with in vivo models. Therefore it is challenge to contribute to these issues with alternative animal-free approaches.

Questions to consider:

- Sensitive windows such as perinatal exposure?
- Chemicals that exacerbate sensitisation reactions?
- Sensitivity for responses to allergens: Age? Gender? Genotype?

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3.5 AXLR8 Scientific Panel Recommendations for Future EU Research & Innovation Funding

The workshop concluded with an in camera (closed) meeting of the AXLR8 Scientific Panel aimed at refining a strategy and roadmap for future EU research in this area, with a clear focus on advancing the 'Europe 2020' goals of addressing major societal challenges through high-impact, results-driven research and robust integration of key technologies in the field of health (and environmental) protection.

There was a general view within and among breakout groups that limitations intrinsic to conventional high-dose in vivo studies limit their relevance and utility as tools for modern safety assessments aimed at protecting and improving human health (e.g., in relation to nanomaterials, endocrine disrupters, and environmental chemicals), and that the way forward requires a shift towards a pathway-based paradigm for safety assessment. In particular, assessment of a substance's toxic 'mode of action' is considered by the AXLR8 Scientific Panel and other authorities to be a cornerstone of '21st century' safety assessment. Development of a robust understanding of the networks of biological pathways—many of which are not yet described in full—and key events associated with chemical toxicity can feed back into the innovation cycle to support 'greener', biocompatible chemistries, and can contribute to the study and treatment of human diseases[1], guiding research on fundamental biology and feeding into the product innovation cycle. By focusing on priority diseases with integration of human patient data, biomonitoring of healthy and diseased populations, and other modern exposure assessment tools, it should be possible to better understand population diversity and susceptibility, and perhaps achieve a closer alignment between human health and environmental risk assessments. Opportunities for synergistic partnerships between EU projects with high-impact initiatives such as Germany's Virtual Liver project, the Japanese METI-NEDO High Throughput Assay Systems project and the US Virtual Embryo, Tox21, and NexGen programmes were noted.

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[1] Gohlke JM, Thomas R, Zhang Y, et al. Genetic and environmental pathways to complex diseases. BMC Syst Biol. 2009; 3, 46-61.

Workshop participants underlined the importance of the higher level of human-relevant biological understanding that can be achieved by means of an integrated pathway and modelling approach to safety assessment, but noted that a substantial investment in targeted interdisciplinary research and related infrastructure will be required to fully develop each of the key 'building blocks' and demonstrate their functional integration before such benefits can be fully realised. It was also noted that early and active interactions with regulatory authorities, regulated industry, and civil society stakeholders will be necessary to achieve timely acceptance and integration of new testing tools and strategies as part of an evolutionary

shift in the safety testing and assessment paradigm.

During its in camera session aimed at developing an innovative toxicity testing roadmap for consideration under the forthcoming Common Strategic Framework for Future EU Research and Innovation Funding, the AXLR8 Scientific Panel noted the substantial progress that has been made in Europe in the development of 'alternative testing strategies' as a product of funding by DG Research & Innovation under FP6 and FP7 (Figure 1). The SEURAT-1 initiative was recognised as an important step towards new experimental and computational approaches to safety testing and assessment, and as a
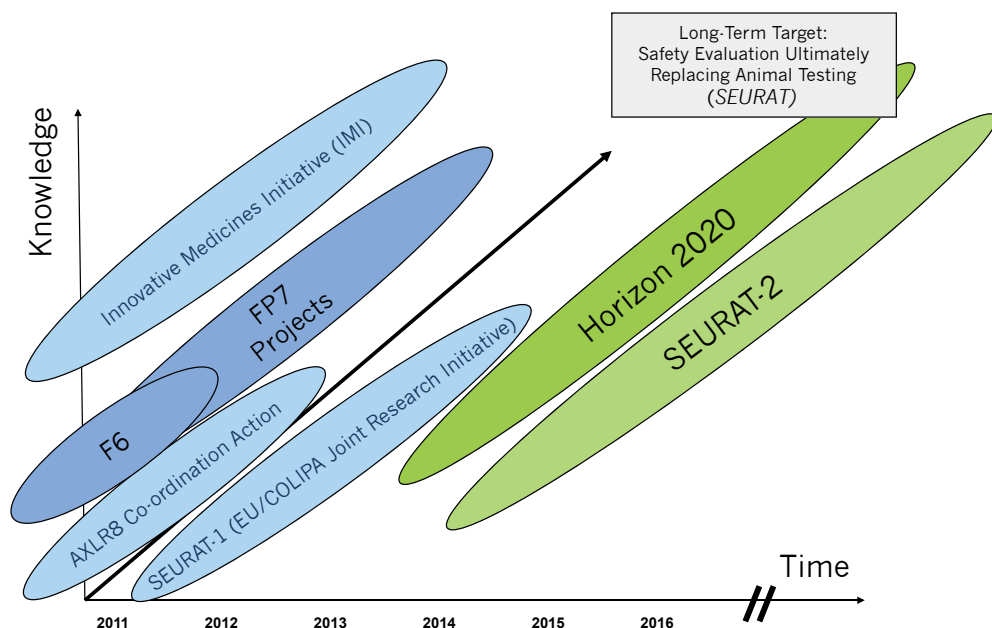

Figure 1. Illustration of the funding activities of the European Commission DG Research & Innovation within the Health theme of FP6 and FP7 in the context of policy needs, industry requirements and scientific challenges to develop 'alternative testing strategies for replacing, reducing and refining the use of animals in research'.
■ programmes currently funded via FP6/FP7 ■ future funding programmes
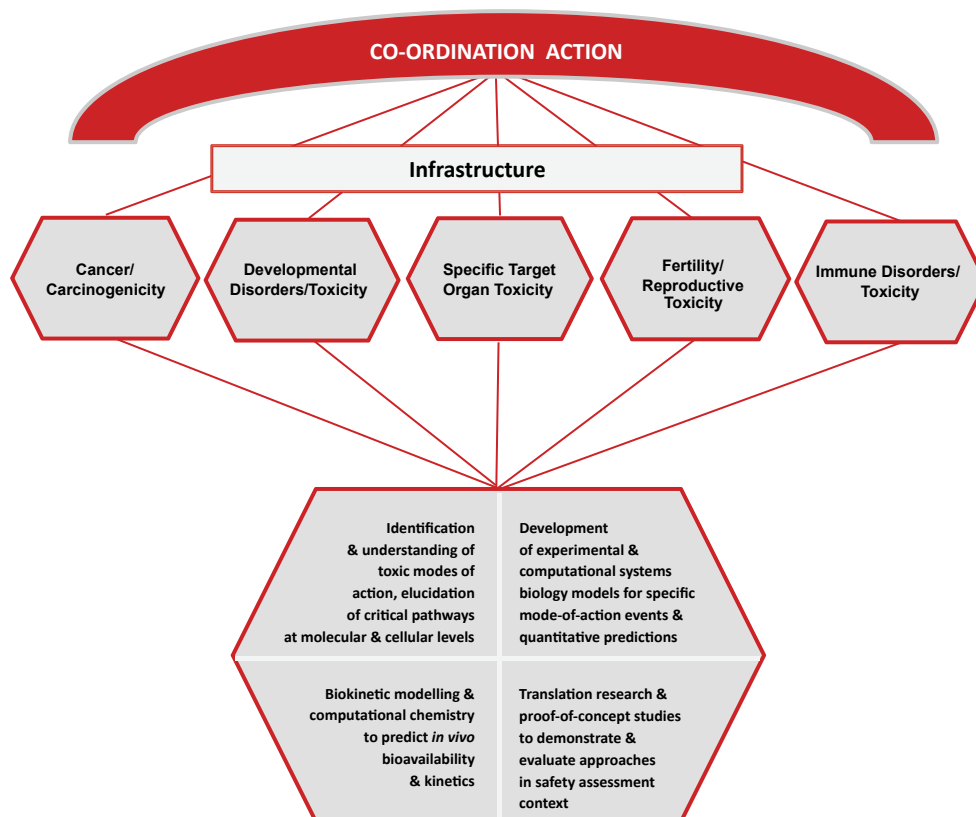
Proposed Structure of 'SEURAT-2'

Figure 2. Illustration of the proposed SEURAT-2 structure. Six large-scale clusters encompassing five human health effect areas together with cross-cutting infrastructure would be developed under the direction of a central co-ordination action. There should be a strong focus in all clusters on core 'building blocks', illustrated here as four distinct projects/research areas; however, the exact number of projects per cluster should be determined on a case-by-case basis.

public-private partnership between the Commission and regulated industry, it represents a promising new funding model. Indeed, COLIPA's direct financial support for this initiative was highly welcomed and favoured over, e.g., in-kind contributions from the private sector.

Structurally, the Scientific Panel welcomed the 'cluster'-type design of SEURAT-1, i.e., multiple project-level 'building blocks'

organised around a central co-ordinating action (Figure 2). This approach facilitates intense scientific exchange and supervision of the research projects/areas (These objectives could also be pursued through creation of a centralised and highly networked 'EU Centre for Molecular Toxicology', where scientists from different disciplines could work at one location toward the same well-defined aim, and through which research and training

could be co-ordinated in a focused way. Work not covered by the EU Centre could be subcontracted to external research groups or clusters as appropriate.) and governance of the cluster. Procedurally, it was recommended that up-front co-ordination and development of project plans and consortia around a central unifying vision—including clear scientific objectives and tangible milestones and deliverables at both project and cluster levels—is essential to ensure strategic alignment within and across projects and cohesion at cluster-level. Procedurally, it was suggested that the existing scientific experts panels could begin now with the development of a detailed roadmap to innovative toxicity testing within the scope of the pathway paradigm. It was also recognised that project co-ordination at cluster-level requires a permanent secretariat run by a group of experts with a multitude of skills at administrative, organisational and scientific levels. Additional administrative instruments to support and enforce cluster-level interactions should be explored in future programmes in areas such as data and knowledge exchange, management and exploitation of intellectual property, and communication and dissemination of research results.

The AXLR8 Scientific Panel considered that the fields of systems toxicology and medicine are primed to advance by a quantum leap, and the EU—as a leading innovator in the area of health research funding aimed at advancing the science of safety testing—is well positioned to play a major role in this dynamic and rapidly evolving research area. Not since the Human Genome Project has the EU been presented with such a tremendous opportunity to contribute to world-class scientific breakthroughs. Indeed, mapping the human 'toxome' is directly analogous to the human genome mapping of the 1990s, and has the potential to be a 'game changer', with substantial benefits foreseen in the areas of public health and environmental protection, economic growth and competitiveness, and animal welfare. Thus, to build on the momentum of successful FP6/7 projects such as ReProTect and Sens-it-iv, and to cover the full spectrum of health and toxicity concerns, the experts concluded that it would be essential to extend SEURAT-1 to its next phase, with integration of all relevant aspects of systems medicine into the core research strategy.

Recommendations for Future EU Research & Innovation Funding

The AXLR8 Scientific Panel recommends swift and decisive action to develop an 'innovation flagship-level' interdisciplinary research effort that builds upon the results of European FP6/7 projects and the emerging results of SEURAT-1, but on a much larger scale given the magnitude of work that is still needed to achieve a full paradigm shift in toxicological safety assessment. The general concept is illustrated in Figures 2 and 3, and is denoted here as 'SEURAT-2'. Taking into account the positive experience of SEURAT-1, SEURAT-2 should be established as a public-private partnership

between the Commission, Member States and regulated industry.

As successfully introduced in SEURAT-1, the key element of SEURAT-2 should be the 'cluster', comprising a group of typically 4-6 individual research projects focused on a particular area. A total of 6 clusters should be funded, organised around five priority health concerns, i.e., cancer/carcinogenicity, fertility/reproductive toxicity, developmental disorders/toxicity, specific target organ toxicities, and immune disorders/toxicity (including sensitisation), which have been identified elsewhere as requiring additional research resources (ADLER ET AL, 2011). An additional cluster is envisaged to address infrastructure and servicing needs, including knowledge management, high-throughput screening platforms, bioengineering, communications, training and outreach. Overall management of the six clusters should be handled by a central co-ordination action. *Based on the SEURAT-1 model, a funding level of 50 million € per cluster could be envisioned, and taken together with a co-ordination action with 3-4 full-time personnel, a total budget of 325 million € would seem appropriate.*

In contrast to previous research, there should be a strong focus in all clusters on the following cross-cutting themes and development of the core 'building blocks' of technical capabilities and models for a common toolbox:

• Identification and understanding of toxicological modes-of-action associated with adverse health effects and disease in humans (Identifying modes of action in other species, e.g., rodents, could be useful for understanding the mechanistic basis for species differences and for bridging the gap between emerging human in vitro data and the results of 'legacy' *in vivo* experiments.), including elucidation of critical perturbations/pathways at the molecular and cellular levels.

• Development of experimental, theoretical and computational models that capture specific mode-of-action events at different scales (molecular, cell, tissue, organ, organism), underpinned by a systems biology approach to integrate models and make quantitative predictions.

• Expansion and refinement of 'physiologically-based biokinetic' (PBBK) modelling and computational chemistry methods to predict in vivo bioavailability, biotransformation and bioactivity of exogenous chemicals.

• Translational research and proof-of-concept activities to realise fit-for-purpose methods and tools for toxicological hazard and potency prediction, and the demonstration and evaluation of these in a safety assessment context.

It is recommended that the SEURAT-2 central co-ordination action would be responsible for articulating initial scientific objectives, milestones and deliverables at both cluster and project levels. To achieve the optimal collection of projects within a cluster, it may be necessary to carry out two successive peer reviews, i.e., the first

focusing on scientific excellence and then on programmatic relevance at cluster-level, in order to maximise potential synergies both within and between projects and clusters. As projects are established, contract agreements should be put in place to clearly define responsibilities and relationships within and among projects, including at the cluster level. Consideration should be given to the use of contracts as opposed to grant agreements in some cases for specific research or service needs. The co-ordination action should establish an independent scientific panel to continually monitor progress at both project and cluster levels, stimulate communication between projects and clusters, define and co-ordinate future tasks and long-range planning of the cluster, and ensure general accountability of all partners toward the common goals. The scientific panel should include the co-ordinators of all projects and further external experts, including international experts. The running of the co-ordination action should be managed by a permanent administrative and scientific secretariat. It is recommended that the role of the co-ordination action be reinforced with effective tools to ensure alignment among projects and enforcement of milestones and deliverables, and that sufficient flexibility be provided to make mid-stream course corrections as needed, for example by bringing in new projects mid-stream when a gap is identified.

'Value added' collaborations among established research teams (e.g., the US Tox21 initiative, Japanese institutes, etc.) in key areas should be encouraged to share the workload, develop synergies without duplication, and together reach for an ambitious, global objective that would be impractical to pursue on a regional basis. Mechanisms to enable the sharing of data and transparency in general will be necessary to build confidence in emerging methodologies. This should allow for recruitment of international partners as appropriate, as well as joint funding calls with Member State and international agencies and funding bodies. The European Commission's Joint Research Centre could make an important contribution owing to its extensive programme in safety assessment methodology and its role in translating scientific results into support to policy-making. Targeted multidisciplinary partnerships should also be encouraged, given that a solution for more predictive and animal-free safety assessment needs the mobilisation of the best scientists in their fields, many of whom would not traditionally apply their work to toxicology. Funding for SEURAT-2 should, as a matter of principle, be used to support research that does not involve the use of living animals.

In conclusion, the AXLR8 Scientific Panel and Consortium believe that the research roadmap outlined above represents the most appropriate path forward to modernise not only safety science, but the wider field of human biomedicine, for the common goals of improved health and consumer protection, innovation and economic growth, environmental sustainability, and animal welfare.

Directory of Projects & Co-ordinators

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START-UP	Vera Rogiers	vrogiers@vub.ac.be	179
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Virtual Liver	Jan Hengstler	hengstler@ifado.de	218
VITROCELLOMICS	Carl-Fredrik Mandenius	cfm@ifm.liu.se	188

Glossary of Terms

2D/3D	Two-dimensional / three-dimensional
3Rs	Replacement, reduction and refinement of animal use
CARDAM/VITO	Centre for Advanced R&D on Alternative Methods at the Flemish Institute for Technological Research
CLP	EU Regulation on the Classification, Labelling and Packaging of Substances and Mixtures
CV	Coefficient of variation
CYP	Cytochrome P450 enzymes
DG R&I	European Commission Directorate General for Research and Innovation
EC	Effect concentration
ECHA	European Chemicals Agency
ECVAM	European Centre for the Validation of Alternative Methods
EPA	United States Environmental Protection Agency
ESC	Embryonic stem cells
FP6/FP7	6th and 7th EU Framework Programmes for Research and Technology Development
GHS	United Nations Globally Harmonised System of Classification and Labelling of Chemicals and Mixtures
GLP	Good laboratory practices
HSI	Humane Society International
HTS	High-throughput screen
JRC	European Commission Joint Research Centre
LOEC	Lowest observed effect concentration
mg/kg	Milligrams per kilogram of body weight

NO(A)EL	No observed (adverse) effect level
NP	Nano-particle
NRC	United States National Research Council
OECD	Organisation for Economic Co-operation and Development
PB(B/P)K	Physiologically-based (bio/pharmaco)kinetic model
(Q)SAR	(Quantitative) structure-activity relationship model
R&D	Research and development
REACH	EU Regulation on the Registration, Evaluation and Authorisation of Chemicals
SOP	Standard operating procedures
TG	Test guideline
WP	Work package

Country Abbreviations

Austria	AT	Latvia	LV
Belgium	BE	Lithuania	LT
Bulgaria	BG	Luxembourg	LU
Canada	CA	Malta	MT
Cyprus	CY	The Netherlands	NL
Czech Republic	CZ	Norway	NO
Denmark	DK	Poland	PL
Estonia	EE	Portugal	PT
Finland	FI	Romania	RO
France	FR	Slovakia	SK
Germany	DE	Slovenia	SI
Greece	GR	Spain	ES
Hungary	HU	Sweden	SE
Ireland	IE	Switzerland	CH
Italy	IT	United Kingdom	UK
Japan	JP	United States	US

It is the aim of AXLR8 to lay the groundwork for a transition in toxicology toward a more pathway-based' *in vitro* and computational approach through enhanced networking and collaboration among scientists, regulators, and other key stakeholders at European and international levels. To achieve this goal, AXLR8 organises annual workshops to bring together the scientific community to discuss the progress of ongoing projects, to identify knowledge gaps, and to propose a scientific roadmap for future EU research.

At the second AXLR8 workshop (AXLR8-2) in 2011, progress on alternatives was reported by FP6/7-funded Health and Environment projects, as well as EU Member State 3Rs centres and international initiatives. A key objective of the second AXLR8 workshop was to develop recommendations for priority research under the forthcoming 'Horizon 2020' EU funding programme. Conclusions arising from the workshop and from discussions of the AXLR8 Scientific Panel are as follows:

)(There is a need for a large-scale, interdisciplinary effort based on a public-private partnership model to further develop the pathway paradigm

)(A key element is functional integration of separate research 'building blocks'
Clusters of 4-5 individual research projects should be organised around priority health concerns, together with an infrastructure cluster and an overarching co-ordination action

)(There should be a strong focus in all clusters on the following cross-cutting building blocks:

- Identification and understanding of toxicological modes-of-action associated with adverse health effects, including elucidation of critical pathways
- Development of models that capture specific MOA events at different scales, underpinned by a systems biology approach to integrate models & make quantitative predictions
- Expansion & refinement of PBPK modelling & computational chemistry methods to predict *in vivo* bioavailability, biotransformation & bioactivity
- Translational research & proof-of-concept activities to realise fit-for-purpose methods & tools for hazard/potency prediction & demonstration of these in a safety assessment context.

Given the substantial and increasing global investment in research aimed at developing new safety assessment methods and implementing the '3Rs' in toxicology, there is a recognised need for better coordination in this research area. In response to this demand AXLR8 has proven that it can provide the tools for effective real-time dialogue, information exchange, problem solving, and international co-operation.