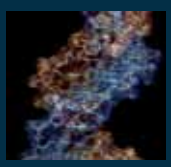
AXLR8

ALTERNATIVE TESTING STRATEGIES PROGRESS REPORT 2010

Replacing, reducing and refining use of animals in research



SEVENTH FRAMEWORK PROGRAMME

Health Biotechnology

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The AXLR8 Consortium

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ALTERNATIVE TESTING STRATEGIES PROGRESS REPORT 2010

Replacing, reducing and refining use of animals in research

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EXECUTIVE SUMMARY

This publication is the third in a series of reports summarising the progress of research to advance the "3Rs" concept (**R**eplacement, **R**eduction and **R**efinement of animal tests) in Europe funded by the European Commission Directorate General for Research and Technology Development (DG-RTD) within the Health theme of the Sixth and Seventh European Research Framework Programmes (FP6 and FP7, respectively) in the context of policy needs, industry requirements, and scientific challenges. This report, prepared by the FP7 coordination action **AXLR8** (pronounced "accelerate") is a follow-up to the 2008 and 2009 European Commission publications, *Alternative Testing Strategies—Replacing, reducing and refining use of animals in research.* It focuses on progress achieved over the last year and provides an outlook on potential future research strategies and priorities.

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** has appointed a Scientific Panel comprised of coordinators of FP6/7-funded 3Rs projects and external experts from academia, regulatory authorities, and regulated industry. To serve its stakeholders, **AXLR8** organises annual workshops to bring together the scientific community to discuss the progress of ongoing projects, to identify knowledge gaps, and to recommend strategic priorities for future EU funding calls.

This *2010 Progress Report* reflects the activities and deliverables of the **AXLR8** project during its first year of operation.

In Chapter 1, the objectives and initial deliverables of the **AXLR8** project are

It is considered by the AXLR8 Scientific Panel that near-term priority research to advance human safety testing and 3Rs mandates should emphasise the following:

- Definition, identification and categorisation of key biological pathways in human toxicology
- Development of an ontology and related taxonomy and vocabulary related to toxicity pathways and adverse effects
- 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.

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.

described, including the first scientific workshop and public Info Forum.

Chapter 2 consists of annual progress reports, and in some cases final reports, prepared by the coordinators of FP6/7-funded projects. As in the previous annual report, these projects have been grouped under the headings "cell-based technologies, integrated testing strategies, 'omics, bioinformatics and computational biology, computational modeling and estimation techniques, high throughput techniques, and forums and workshops". Since these projects have all been described in detail in the 2008 and 2009 annual reports, previously published content will not be repeated here.

Chapter 3 contains brief reports from 3Rs initiatives from EU member states and Japan, together with updates from US colleagues working on the implementation of the National Research Council (NRC) vision of "21st century toxicology", and a view from the Organisation for Economic Co-operation and Development (OECD) Test Guidelines Programme.

Chapter 4 reflects the views of **AXLR8** Scientific Panel members articulated at the **AXLR8-1** workshop in relation to

progress being made by FP6/7 projects and on implementing the vision of pathway driven molecular toxicology proposed by the US NRC. The **AXLR8** Scientific Panel concluded that substantial progress is being made in Europe in the development of alternative test methods and integrated testing strategies. Examples such as the ReProTect project show that feasibility studies can be most useful to demonstrate the potential of new non-animal test systems. The main sections of this chapter cover critical discussions of the current European research projects, of international paradigms and initiatives, and finally of the framework for laying the groundwork for a toxicity pathway-based research strategy in Europe. This knowledge is relevant not only in relation to safety assessment of chemicals and products, but also for other scientific areas such as systems medicine.

Chapter 5 presents thoughts on a way forward and next steps for European research. Thus, the new elements of the *Progress Report 2010* are setting 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 coordinated research and technology development, which is a major objective of the **AXLR8** project.

1 INTRODUCTION

To date almost 150 million € in funding has been provided under the EU's 6th and 7th Research Framework Programmes to advance the development and validation of 3Rs methods and testing strategies for regulatory purposes.

These funding activities have been "policy-driven" by the former Directive 86/609/EEC (soon to be replaced by Directive 2010/63/EU) for the protection of animals used for 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 legislative mandates to replace regulatory toxicity testing in animals with non-animal approaches. To achieve this goal, a number of ambitious projects have been launched, in which scientists from industry, academia and/or government have embarked on programmes of work based on replacing animal use on the level of classical toxicological endpoints such as acute and reproductive toxicity.

In addition, a variety of *in vitro* and computational toxicology initiatives are under way across EU member states, North America, Asia, and elsewhere. Monitoring the scope and progress of these multidisciplinary activities 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 FP7 coordination project **AXLR8** is supporting this process by providing up-to-date information to the European Commission DG-RTD 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 fast-paced 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. 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 such as the US National Research Council[1] and others[2-5] to call for shift in toxicity testing towards the elucidation of "toxicty pathways" at the cellular level—an approach commonly referred to as "21st century toxicology".

Recognising the growing need of a focal point for monitoring the increasing number of 3Rs and related multidisciplinary research initiatives worldwide, the United Kingdom branch of Humane Society International (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 coordinators of EU FP6/7 3Rs 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 to advance the science of safety testing. 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 Euro-

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[1] *Toxicity Testing in the 21st Century: A Vision and A Strategy*. Washington, DC: National Academies Press (2007).

[2] Berg N, De Wever B, Fuchs HF, et al. (2010). Toxicology in the 21st Century—working our way towards a visionary reality. Willemstad, NL: In Vitro Testing Industrial Platform.

[2] Ankley GT, Bennett RS, Erickson RJ, et al. (2009). Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. E*nviron Toxicol Chem.* 29, 730-41.

[3] *New Perspectives on Safety*. Brussels: EPAA (2008).

[4] *A National Toxicology Program for the 21st Century: A Roadmap for the Future*. Research Triangle Park, NC: NTP (2004).

pean and global levels. To this end, the following specific objectives will be pursued:

- Monitoring of DG-RTD-funded FP6/7 3Rs research projects and publication of annual progress reports.
- Establishment of a Scientific Panel with experts from the EU corporate, governmental and academic sectors, together with 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 FP6/7 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 working to develop 21st century approaches to safety testing in order to encourage and lay the groundwork for more efficient acceptance of these 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 coordinators of EU-funded FP6/7 projects and independent experts in toxicology (*in vitro*, *in silico* and mammalian), systems biology, 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 1.

AXLR8-1 Workshop

The first **AXLR8** workshop was held in Potsdam, Germany from 31 May to 2 June 2010. The programme is available online at *AXLR8.eu/workshops*. Participation was limited to the coordinators of research projects funded through the FP6/7 Health programme, invited representatives of 3Rs centres and initiatives from EU member states and Japan, leading US scientists working to advance the vision of "21st century toxicology", members of the **AXLR8** Scientific Panel and Consortium, and representatives of the European Commission DG-RTD and Joint Research Centre.

The first day-and-a-half of the workshop was reserved for presentations from the coordinators of EU-funded FP6/7 projects to highlight progress achieved during the year 2009. Presentations were also given by representatives from 3Rs centres in

Table 1. Members of the AXLR8 Scientific Panel.

European experts

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

International experts

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

Representatives of EU FP6/7 3Rs Research Projects

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

Austria (ZET), Belgium (CARDAM), Finland (FICAM) and Germany (ZEBET), as well as by government and academic scientists from Japan and the United States. The final day of the workshop was reserved for a meeting of the **AXLR8** Scientific Panel to reflect on the presentations, to discuss the current state-of-the-art, identify knowledge gaps and opportunities for synergies, and to propose priorities for future research.

AXLR8 Info Forum

On 1 September 2010, **AXLR8** hosted a half-day public information forum in Linz, Austria, in conjunction with the Linz 2010 congress, co-hosted by the European Society for Alternatives to Animal Testing and the European Society for Toxicology *In Vitro*. The **AXLR8** forum attracted more than 100 scientists from academia, industry, government institutions, animal welfare organisations, as well as students. Following an introduction by the **AXLR8** Consortium, four state-of-the-art lectures were given by coordinators of the EU FP-funded projects PREDICT-IV, Sens-it-iv and VITROCELLOMICS, and by a representative of the US ToxCast™ and Tox21 initiatives. These presentations are available online at *AXLR8.eu/workshops*. Following the plenary presentations, a panel discussion explored the strengths and limitations of various international research and funding initiatives, legislative and other drivers for developing non-animal testing strategies, and opportunities for transatlantic collaboration.

Progress Report 2010

Building upon the 2008 and 2009 progress reports published by the European Commission DG-RTD, *Progress Report 2010* provides an annual update on the activities and achievements of research funded under the FP6/7 Health programme. In addition, this report takes into account the results of the **AXLR8-1** workshop and Info Forum. The project updates in Chapter 2 have been drafted by the coordinators of the respective FP6/7 projects. Chapter 3 contains contributions from EU member state 3Rs centres and contributions from colleagues from Japan and the United States who participated in the **AXLR8-1** workshop and Info Forum. Chapter 4 summarises the discussion and recommendations from the meeting of the **AXLR8** Scientific Panel at the **AXLR8**-1 workshop, and Chapter 5 offers thoughts on the way forward in implementing integrated testing strategies based on 3R methods. The **AXLR8** consortium is grateful to members of the **AXLR8** Scientific Panel and to participants in the Info Forum for their thoughtful contributions, which are reflected in this report.

AXLR8 Website

The website *AXLR8.eu* provides "one stop shopping" for links to FP-funded 3Rs 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.

2 PROJECTS SUPPORTED BY THE EU HEALTH PROGRAMME

The projects supported by the EU Health programme covered in this chapter are grouped according to the following topics, in line with the respective technologies and approaches:

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

Forums and workshops are exclusively supported by the funding scheme of "Specific Support Actions" (SSA) covering training, conferences or prospective studies in support of the programme.

The remaining projects are more difficult to group since in some approaches are used, which fit into more than one of the topics. This is particularly true for the large multidisciplinary "Integrated Projects" (IPs), since in some of them cell models are combined with genomics, proteomics, metabolomics, (Q)SAR analysis, and/or biochemical pathway analysis. But some overlapping of subjects also occurs among "Specific Targeted Research Projects" (STREP) and "SME-Specific Targeted Research Projects" (SME-STREP), which are smaller projects focusing on specific research issues and are less multidisciplinary than Integrated Projects.

Table 2 gives an overview of the projects according to their common features.

Table 2. Overview of FP6/7 3Rs projects.

Project technologies & approaches	Projects	Framework programme	Funding scheme
Cell-based technologies	ReProTect	FP6	IP
	VITROCELLOMICS	FP6	STREP
	MEMTRANS	FP6	STREP
	EXERA	FP6	SME-STREP
	INVITROHEART	FP6	SME-STREP
	LIINTOP	FP6	SME-STREP
	ARTEMIS	FP6	SME-STREP
	ESNATS	FP7	IP
Integrated testing strategies	ACuteTox	FP6	IP
-omics, bioinformatics & computational biology	Sens-itiv	FP6	IP
	carcinoGENOMICS	FP6	IP
	PREDICT-IV	FP7	IP
Computational modelling & estimation techniques	OpenTox	FP7	STREP
High-throughput techniques	COMICS	FP6	SME-STREP
	NanoTEST	FP7	STREP
Forums & workshops	ForInViTox	FP6	SSA
	InViToPharma	FP6	SSA
	START-UP	FP7	SSA

2.1 Cell-based technologies

RePro Tect

Development of a novel approach in hazard and risk assessment of reproductive toxicity by a combination and application of *in vitro*, tissue and sensor technologies

Contract number:	LSHB-CT-2004-503257
Project type:	Integrated Project (FP6)
EC contribution:	€ 9 100 000
Starting date:	1 July 2004
Duration:	66 months

Website: http://www.reprotect.eu

Background

ReProTect was an integrated project that began in July 2004 and ended in December 2009. The project was sponsored by the European Commission through FP6 with a total budget of 13.2 million € (see reference 1 and *ReProTect.eu*). The consortium was composed of 33 European partners from academia, small- and medium-sized enterprises (SMEs), governmental institutes, and others. ReProTect was the first proposal that aimed at improving consumer and patient safety by using sophisticated *in vitro*/*in silico* tools, and at the same time contributing to the replacement, reduction and refinement of animal experimentations as described as "3Rs" principle. The ReProTect application was using the newly established instrument of Integrated Projects within FP6. Due to implementation of the Registration, Evaluation and Authorisation of Chemicals (REACH) regulation, a dramatic increase in animal consumption for toxicity safety testing is expected. It is estimated that around 5000 chemicals will require testing for reproductive and developmental toxicity. As a consequence, millions of animals will be sacrificed for the identification of reproductive toxicants. Moreover, it has been calculated that roughly 60% of the total costs of the REACH exercise will be spent for reproductive toxicity studies. Therefore, the integration of alternative tests in testing strategies that will use existing information from animal studies and integrate additional information from alternative tests for reproductive toxicity (*in vitro* tests, QSARs and read-across approach-

es) is strongly encouraged. At the same time, the 7th Amendment of the Cosmetics Directive mandates the phasing out of animal experimentations for reproductive toxicity testing for cosmetic ingredients. On this background, the overall aim of ReProTect was to develop and optimise *in vitro* assays and *in silico* methods to become part of a testing battery that will provide detailed information on adverse effects of chemicals and other agents to the mammalian reproductive cycle. The ReProTect project was organised into 4 research areas (R.A.) as is schematically outlined in Figure 1.

Historically, the ReProTect project was brought to life by Herman Koëter (at that stage Scientific Director of the European Food Safety Authority, EFSA) and Thomas Hartung, who was head of the European Center for Validation of Alternative Methods (ECVAM) at that time. After internal discussions, ECVAM had taken lead to set up the project with the aim to make sure that the tests in the area or reproductive toxicity are sufficiently developed and ready to enter into the validation process. For starting up the proposal, ECVAM organised a first meeting with external senior scientists with subsantive experience in reproductive toxicity testing covering various stakeholder organisations. The industrial sector was represented by Peter Gelbke (BASF); Herman Koëter (EFSA) and Alberto Mantovani (Instituto Superiore di Sanita', Rome, Italy) covered regulatory aspects in alternatives, and Horst Spielmann (ZEBET), Thomas Hartung and Susanne Bremer (both ECVAM) spoke for the needs in alternative testing. During this meeting, the structure of ReProTect into research

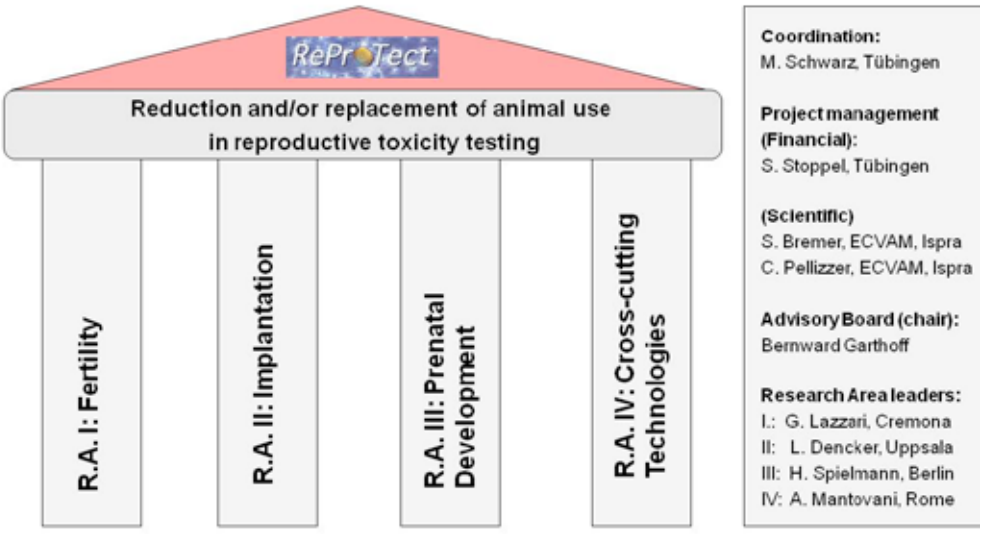


Figure 1. Organisation of ReProTect into Research areas (R.A.) I-IV.

Table 1. ReProTect Advisory Board members.

Participant name	Affiliation at time of entry to the project	Country
Garthoff, Bernward	Bayer Cropscience AG	DE
Amcoff, Patric	OECD Environment, Health and Safety Division	FR
Fielder, Robin	Dept. of Health, Environmental Chemicals Unit PH5D	UK
Gabrielson, Karin	ecopa, Swedish Fund for Research Without Animal Experiments	SE
Gelbke, Peter	BASF Aktiengesellschaft	DE
Koëter, Herman	EPSA, European Food Safety Authority	BE
Liminga, Ulla	Medical Products Agency	SE
Meyer, Otto	Danish Institute for Food and Veterinary Research	DK
Rogiers, Vera	V.U.B., Vrije Universiteit Brussel	BE
Van Cauteren, Hermann	Tibotec BVBA	BE
Vanparys, Phillippe	J & J Research and Development, Genetic and in vitro Toxicology	BE
Vericat, Joan Albert	Neuropharma, S.A.	ES

areas, their leaders, as well as various scientific objectives were discussed and defined. In addition, the involvement of other relevant stakeholder organisations as part of the advisory board were identified.

The following scientists were selected as heads of research areas and were actively involved in the planning of the project from the very beginning on: R.A. I, Fertility: Giovanna Lazzari (Aventea, Cremona, Italy); R.A. II: Implantation, Lennart Dencker (Uppsala University, Sweden); R.A. III, Prenatal Development: Horst Spielmann (ZEBET, Berlin, Germany); and R.A. IV, Cross-cutting Technologies: Alberto Mantovani (Istituto Superiore di Sanità, Rome, Italy). In collaboration with the R.A. leaders, new partners providing additional expertise in reproductive toxicology were identified and contacted (Table 1). Most of the original project proposal was written by Susanne Bremer at ECVAM.

After the proposal of the project was successfully evaluated, it became clear that it could not be coordinated by ECVAM due to a potential conflict of interest, since ECVAM, as part of the Joint Research Center (JRC), was directly financed by the European Commission. Therefore, the project was handed over to an independent coordinator with toxicological expertise. In early 2004, Michael Schwarz, head of the Department of Toxicology at the University of Tübingen, agreed to take over this charge. The kick-off meeting of ReProTect was then organised at the JRC in Ispra, Italy, on 19-21 July 2004. At that time, only two

researchers of R.A. II, Lennart Dencker and Lisbeth Knudsen, were already partners in the project. The other members of this research area were selected through an open call and started their work in year three of the ReProTect project.

During the entire period of ReProTect, the project obtained scientific advice through an independent advisory board (termed "Supervising Board", S.B.), which was chaired by Bernward Garthoff (Bayer Crop Science). He was nominated as a stakeholder in his function as vice-chair of the European Consensus Platform on Alternatives (ecopa) together with the chair, Vera Rogiers, and Karin Gabrielson as nominee of animal welfare organisations (Table 1).

The basic idea in ReProTect was to break down the reproductive cycle into its biological components in order to cover as many of the fundamental aspects of mammalian reproduction as possible. The various tests to be developed/optimised were therefore selected to cover key aspects of male and female fertility (R.A. I), implantation (R.A. II), and prenatal development (R.A. III), while most of the assay systems in R.A. IV aimed at detecting adverse effects of test compounds on the endocrine system. The selection of relevant target cells/biological mechanisms as well as the review on the performance of tests was supported by a series of strategic internal and external workshops. The reproductive cycle is schematically illustrated in Figure 2, which also graphically delineates the position of each of the more than 25 tests of the ReProTect project within the reproductive cycle.

An overview on original publications obtained in ReProTect is presented at the end of this report. In addition to these publications, the ReProTect consortium decided to compile all reports on the so far unpublished results obtained by the individual partners in one "Special Issue" to be published in *Reproductive Toxicology*, in order to allow the reader to gain a comprehensive insight into the structure of this large international project and its achievements. (For publications related to the introduction see references 1-5.)

Results

Research Area I: Fertility

R.A. I was focused on fertility and working on the establishment of *in vitro* bioassays for the detection of toxic effects on gametes, the early embryo, and on the different cell types present within the male and female reproductive tracts. Both primary cells and established cell lines have been included in the project and, besides the classical rodent model, a bovine model has been explored for its usefulness as a tool for toxicity screening. The research area includes six work packages (WP), which cover the major biological processes and mechanisms responsible for successful procreation.

WP I.1 Mature spermatozoa

Sperm viability, motility and DNA integrity are hallmarks for sperm function. These

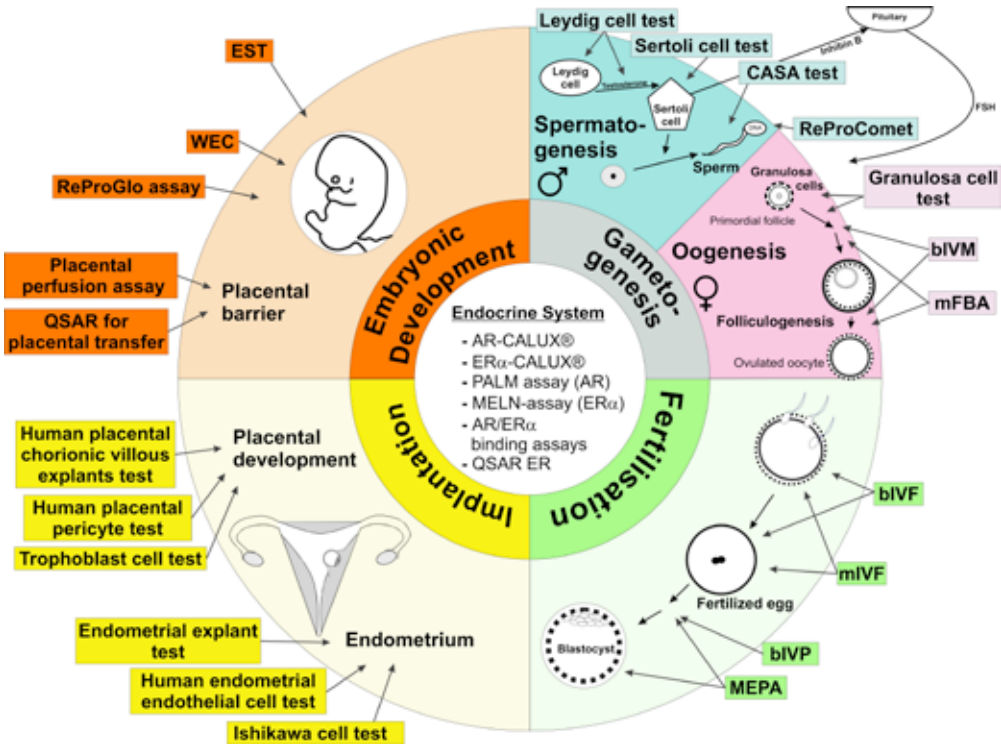


Figure 2. The test systems of the ReProTect project and their applicability position within the mammalian reproductive cycle. The abbreviations are: AR CALUX, androgen receptor (AR)-Chemically Activated LUciferase eXpression (agonist and antagonist); bIVF, bovine *in vitro* fertilisation assay; bIVM, bovine *in vitro* maturation assay; bIVP, bovine *in vitro* pre-implantation assay; CASA, computer assisted sperm analysis; EST, embryonic stem cell test; ERα CALUX, estrogen receptor (ERα)-Chemically Activated LUciferase eXpression (agonist and antagonist); MELN assay, MCF-7 (ER+) stably transfected cells with the estrogen responsive gene ERE-ßGlob-Luc-SVNeo assay (agonist and antagonist); mFBA, mouse follicle bioassay; mIVF, mouse fertilisation assay; MEPA, mouse embryo peri-implantation assay; PALM assay, PC-3-Androgen receptor-Luciferase-MMTV assay; QSAR ER, quantitative structure-activity relationship for the estrogen receptor; ReProGlo, ReProGlo assay; WEC, whole embryo culture assay.

parameters have been investigated on frozen-thawed mature bovine spermatozoa for their ability to reveal chemical toxicity. The Computer-Assisted Sperm Analysis (CASA) test is a simple, fast and consistent method to predict potential impact of chemicals on male fertility using sperm motility and membrane integrity as endpoints. CASA can assess spermatozoa damage via detection of motility, morphology, and sperm count. Spermatozoa must be alive and motile, able to undergo acrosome reaction, and able to deliver an intact male genome into the oocyte to create an embryo with a high chance of completing a full-term healthy pregnancy. Any damage to these properties (motility, plasma membrane integrity, genetic integrity) could hamper its reproductive capacities before or after the fertilisation process.

The use of frozen semen represents an innovative approach and does not involve invasive procedures which compromise animal welfare. Frozen bovine semen is readily accessible and represents a material that can be stored for long periods in liquid nitrogen without losing its biological properties.

Prevalidation of the CASA has been performed in two independent laboratories by Andrea Galli, Istituto Sperimentale Italiano "Lazzaro Spallanzani", Milano, IT, and Ine Waalkens-Berendsen, TNO, Zeist, NL, by testing more than 35 test substances representing chemicals of different classes. The reproducibility between both laboratories was generally acceptable. It turned out, however, that adult bovine sperm are relatively resistant to potentially adverse effects of reprotoxicants, which have been selected as training set which makes the assay appear somewhat less sensitive.

Chemical effects on sperm DNA/chromatin integrity were preliminary assessed by the flow cytometric Sperm Chromatin Structure Assay (SCSA) and the Comet assay. These high-throughput assays are able to detect DNA strand breaks on a cell-by-cell basis *in situ* and, in the case of SCSA, also chromatin structural alterations. Liquid nitrogen-frozen bull sperm were identified as the suitable cellular system for testing alterations induced by chemicals on the sperm DNA/chromatin and *ad hoc* standard operating procedures (SOPs) were set up for this purpose. To detect also chemically-induced pre-mutagenic DNA lesions eventually leading to breaks, a modification of the Comet assay was developed in Marcello Spano's group at ENEA, Rome, IT. The test, termed ReProComet, circumvents the intrinsic repair deficiency of sperm cells in that the assay is supplemented with a protein extract from somatic cells after the chemical treatment. It was shown that the repair enzymes present in the extracts could initiate the removal of adducts on sperm DNA forming strand breaks detectable by the Comet assay. (For publications in WP I.1 see references 6 and 7.)

WP I.2 and I.3 Leydig cells and Sertoli cells

A disturbance of the endocrine system

due to chemical effects on steroidogenesis or due to specific cytotoxic effects on Leydig or Sertoli cells leads to a decreased development of spermatozoa and impaired fertility. Sertoli cells form the basis of the blood-testis barrier and divide the tubular area into adluminal and basal compartments protecting the maturing germ cells from chemical insults. Leydig cells nurture the developing sperm cell. Leydig and Sertoli cells have been exposed to model chemicals in order to identify relevant toxicological targets. In addition, rat testicular fragments in short-term dynamic cultures have been evaluated as a model to study effects on androgenesis.

A new Leydig cell line, BLT1-L17, which responds very well and quite robustly to luteinising hormone (LH) or its analogue, chorionic gonadotropin (hCG), has been characterised by Axel Themmen at the Department of Internal Medicine of Erasmus MC, Rotterdam, NL. The MTT assay was chosen as a general toxicity endpoint, and testosterone production as the Leydig cell-specific endpoint. The BLT1-L17 cells were exposed to 15 chemicals, and the data obtained with this set of test chemicals indicate that the cell line is an excellent candidate for further development into a rigorous test applicable for *in vitro* reproductive toxicity assessment acting via interference with testosterone production.

The response of two rat Sertoli cell cultures (primary cultures and the SerW3 line) to 15 chemicals known to induce testicular damage was evaluated by Anne Tilloy-Ellul at Pfizer, Amboise, France. General cytotoxicity and the secretion of inhibin B were measured. These two endpoints allowed a classification of the chemicals as positive or negative for testicular toxicity. Seven compounds were tested in two different laboratories, and the data were reproducible in both culture systems. In addition, the integrity of tight junctions forming the blood-testis barrier was studied in the SerW3 cell line, providing a new endpoint to study the mechanism of action of testicular toxicants. Finally, experiments with rat testicular fragments have shown that testosterone production is readily detectable inside fragments and follows stimulation with hCG. Compounds that are strongly and moderately toxic on steroidogenesis are detectable in this assay, although a clear concentration-effect relationship could not always be demonstrated. (For publications in WP I.2 see reference 8.)

WP I.4 Meiotically competent oocytes, follicle culture

Mouse follicle culture, *in vitro* maturation and fertilisation of mouse and bovine oocytes, and pre-implantation mouse and bovine embryonic development were the *in vitro* bioassays under investigation in this WP.

Bovine oocytes were used in the laboratory of Giovanna Lazzari, Aventea, Cremona, IT. The objectives of the two-assay systems based on oocyte use were the detection of adverse chemical effects during the processes of oocyte maturation and fertilisation. The toxicological endpoints of the assays are the achievement of

metaphase II and the formation of two pronuclei, respectively. The tests were shown to be robust across replicates, and the toxicological potential derived from the *in vitro* results corresponded well with the effects of the test chemicals *in vivo*. Preliminary prediction models for both tests were generated. The results (EC_{50}-values) obtained for a panel of different chemicals are shown in Figure 3, along with the proposed target structure and mechanism of action of the various agents.

For the majority of agents, the maturation assay turned out to be more sensitive (e.g., cycloheximide), while for others there was no obvious difference between the two assays (e.g., nocodazol). This can be explained by their different mode of action, as schematically outlined in Figure 4. While inhibition of protein synthesis by cycloheximide is only deleterious during the complex phases of oocyte maturation, inhibition of microtubule function by nocodazol inhibits spindle formation in meiotic divisions during oocyte maturation, but also flagella function of the moving sperm. This indicates that the *in vitro* assays provide mechanistic understanding of the mode of action of the test agents, which is a valuable additional information for risk assessment.

The transferability of the bovine maturation test has been achieved by implementing the assay in a second laboratory, namely that of Alberto Maria Luciano at the Università degli Studi di Milano, IT. The inter-laboratory variability and the transferability of the bIVM test were then analysed for a set of eight chemicals, and the statistical analysis of the data obtained from the two laboratories demonstrated

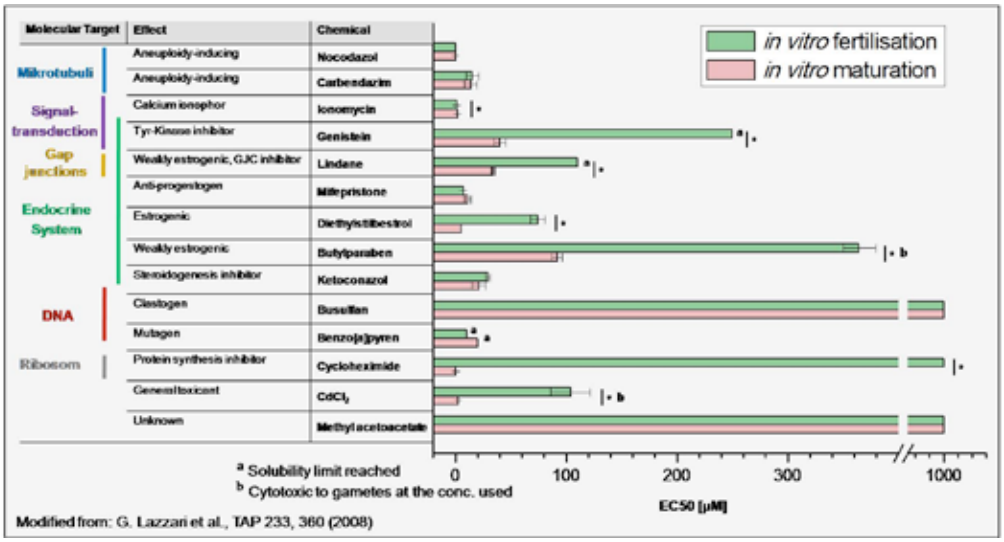


Figure 3. Effect of a panel of test chemicals in the bovine *in vitro* maturation and in vitro fertilisation assays. The presumed target structure and mode of action of the test agents is also indicated.

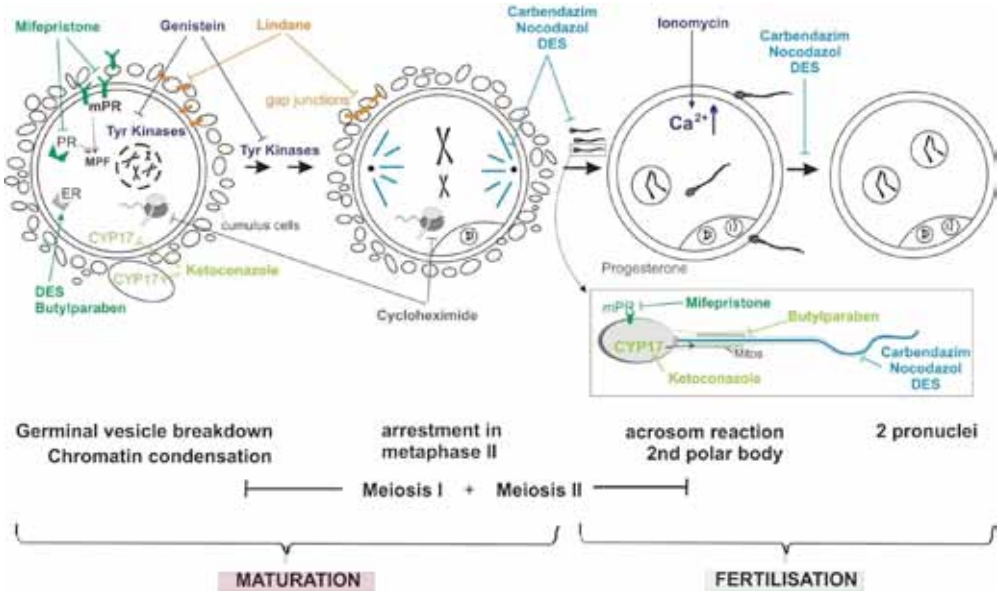


Figure 4. Schematic representation of the processes of maturation and fertilisation with the presumed targets of the test agents used in the bovine *in vitro* tests.

that there was a good concordance of results across the laboratories. Overall, the results show a high reliability of the assay to detect adverse effects on female fertility.

The mouse follicle bioassay (mFBA) was established at EggCentris, Brussels, BE (Rita Cortvrindt). The mFBA is a long-term, multi-parametric organ culture mimicking *in vitro* follicle growth and development from the early preantral stage up to ovulation of the oocyte. This follicle bioassay starts with mechanical isolation of early preantral follicles out of mouse ovaries and initiation of individual follicle culture. On day 1, selection of the *in vitro*-adapted intact early secondary follicles consisting of the oocyte, granulosa and theca cells and an intact basal membrane are exposed to the different experimental conditions. The follicles grow and develop during a 12-day culture period from the preantral stage over the antral stage to pre-ovulatory follicles, with proliferation of the theca cells, on top of which granulosa cells proliferate and differentiate into mural and cumulus cell, enclosing the growing oocyte. Concurrently, P450 enzymes driving steroidogenesis are expressed in a place- and-time dependent manner. The LH-responsive theca cells produce androgens continuously, converted by Cyp19 (aromatase) in the FSH stimulated granulosa cells to estrogens.

Oogenesis is closely linked and dependent to the process of folliculogenesis. The oocyte grows mainly during the preantral and early antral stage of folliculogenesis, by stockpiling proteins and RNA for later use, and as such achieve nuclear competence

(i.e., the capacity to reinitiate and complete the meiotic process). During the late antral stage, the reorganisation of the ooplasmic constituents determines final oocyte quality (i.e., the capacity to support early pre-implantation development/ developmental competence).

At the pre-ovulatory stage (day 12), an ovulatory stimulus will induce secretion of hyaluronic acid with mucification of the cumulus oocyte complex, re-initiation of the meiosis I in the oocyte, and induction of Cyp 17 in the mural granulosa cells, driving a steep increase of progesterone secretion.

The process of folliculogenesis is analysed light microscopically at defined time points (days 4, 8, 12) reflecting the different stages of follicle development, and at the same time steroid measurements in the respective spent media elucidates the steroid profile of the follicle. On day 13, the impact of the ovulatory stimulus is evaluated by the degree of mucification, the oocyte nuclear maturation status (GV, GVBD, PB or deg), and the increase in progesterone in the spent medium.

Subjecting these *in vitro* grown oocytes to an *in vitro* fertilisation procedure, with subsequent culture of the embryo up to the blastocyst stage, allows the characterisation of the developmental capacity of the oocyte.

Upon optimisation and standardisation of the follicle bioassay, the selected set of end parameters proved to be highly reproducible, enabling identification and quantification of the impact of chemicals on the ovarian function/fertility (e.g., cycle disturbances, conception capacity, homeostasis). The effect of more than 15 chemicals on growth and maturation of female germ cells, as well as on the production of different steroids during the culture period, were studied. For a schematic representation of the mFBA see Figure 5A.

The mouse peri-implantation assay (MEPA) developed at EggCentris, Brussels, BE (Rita Cortvrindt) is an *in vitro* bioassay that enables the study of the effect of compounds on the development of the pre-implantation embryo and its capacity to survive upon hatching around the implantation period. The assay is based on the *in vitro* culture of mouse zygotes. The zygotes are cultured in groups of 10 for 7 days, with daily observation and scoring of embryo development. These daily morphological observations allow for pinpointing of potential deviations in the timely regulated pre-implantation embryo (Figure 5B). This bioassay is highly reproducible, with confidence interval of blastocyst development on day 6 between 95.7-97% and a CV of 6.6%. It allows easy determination of the no effect level at the pre-implantation embryo and the characterisation of the sensitive stage of embryo development, indicating the mode of action of the substance. (For publications in WP I.4 see references 9-14.)

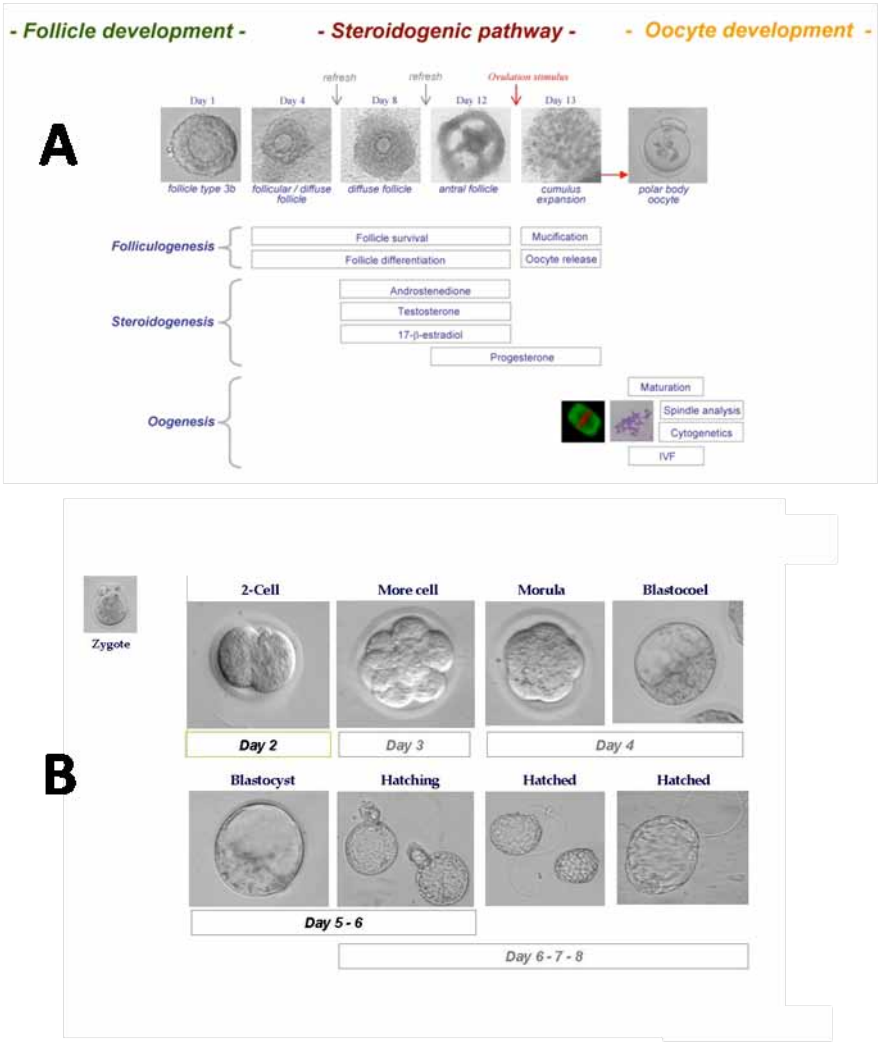


Figure 5. Schematic representation of A: the multi-parametric mouse follicle bioassay and B: The Mouse Embryo Peri-implantation assay (MEPA)

WP I.5 Granulosa/Thecal cells and WP I.6 Folliculogenesis

The aim of the two WPs was to develop test systems able to predict adverse effects of chemicals on female steroidogenesis and folliculogenesis. An *in vitro* screening assay for detection of toxic and endocrine effects of chemical compounds was developed by Ilpo Huhtaniemi at the Imperial College in London, UK, using genetically modified immortalised murine granulosa cells. Granulosa cells are the site of estrogen production in the ovary, and female fertility is dependent on estrogens, amongst others, produced by the ovary. Because various chemical compounds can potentially interfere with granulosa cell endocrine function, it is desirable to have an *in vitro* testing system for screening of such potentially harmful effects. The cell line used (NT-1) is derived from a murine

granulosa cell tumour that developed in a transgenic mouse expressing the SV40 T-antigen under the inhibin-alpha promoter. Endpoints of the assay were progesterone production in the absence of a steroid substrate and estradiol production in the presence of androstenedione substrate. 12 compounds were selected on the basis of their known or potential inhibitory effects on steroidogenesis and reproductive function and then tested in the assay. Unfortunately, however, the toxic effect of most of the compounds was greater that their specific effect on steroidogenesis. For this reason, the assay does not qualify for its adaptation for a wider use.

Johan Smitz from the Vrije Universiteit Brussels, BE, used the *in vitro* mouse follicle assay from EggCentris to screen for potential adverse effects of test agents on steroidogenesis, while evaluating at the same time effects on *in vitro* folliculogenesis and oogenesis. Quantitative determination of the effective concentrations of the principle steroids (estradiol, progesterone, estrone, testosterone and androstenedione) enables definition of the critical concentration of a test chemical that interferes with steroid production and secretion. By testing a series of compounds with known mode of action, the system was found to be sensitive in identifying compounds that affect steroidogenesis in a highly reproducible way. The most sensitive parameters of the assay, established and assessed by Rita Cortvrindt at EggCentris, include follicular growth, differentiation, survival and oocyte maturation, which can all be easily assessed under a normal stereomicroscope, while steroid hormone production was measured in the lab of Johan Smitz by means of radioimmunoassay on conditioned medium. (For publications in WP I.5 and WP I.6 see references 15-17.)

Research Area II: Implantation

WP II.1

This WP explores endometrium/ implantation and oviduct studies, in which cell culture models and explants have been established that represent the endometrium, the abnormalities or dysfunction of which may have detrimental effects on female fertility. The main focus was to analyse effects on the estrogen receptor, on apoptosis and on drug metabolising cytochrome P450 enzymes in the target cells. It turned out that the use of human explants tissue was very difficult to organise and that there was a very high variability in the results obtained with explants tissue.

Alternatively to the human endometrial explants, the human endometrial epithelial Ishikawa cell line was applied by the group of Wolfgang Schaefer, University of Freiburg, DE, to predict effects of chemicals in the human endometrium. This test system is a tissue-specific model to detect estrogenic activity of chemicals which up-regulate progesterone receptor (PR) mRNA in the human endometrium. In the model several chemicals have been analysed: DES, 17ß-estradiol, bisphenol A and nonylphenol up-regulated PR mRNA a dose-dependent manner (Figure 6), whereas progesterone, RU486 and

cadmium chloride (≤10^{-6} M) had no up-regulating effects on PR mRNA and were classified as negative compounds. The Ishikawa model is informative regarding the mode of action of positive tested chemicals and provides guidance for prioritisation for further testing.

In order to clarify whether human endometrial explants may be useful for mechanistic studies, Eva Brittebo and colleagues from the Uppsala University, SE, examined bioactivation and effects of the model endometrial toxicant tamoxifen. This drug is metabolised by various CYP enzymes to pharmacologically active metabolites, but it is also converted to reactive intermediates. The results suggest local bioactivation of tamoxifen in human glandular and surface epithelia, with the epithelial cells being early target sites for tamoxifen-induced cell stress. In addition, the group examined the gene expression of major drug-metabolising CYP enzymes in human endometrial endothelial cells (HEEC), in HEEC co-cultured with stromal cells and in human placenta samples. The studies demonstrated a constitutive gene expression of CYP enzymes, and suggest that the human endometrium and placental membrane have the potential to detoxify or bioactivate drugs and chemicals. However, due to large inter-individual variation and the paucity of fresh endometrial samples, HEEC seem to be less useful for screening assays predicting reproductive toxicity of drugs and chemicals. Nevertheless, human endometrial explants and the expression of the CYP1 enzymes in HEEC may be of interest for mechanistic studies on adverse effects of drugs and chemicals if the cellular and inter-individual variation is taken into account.

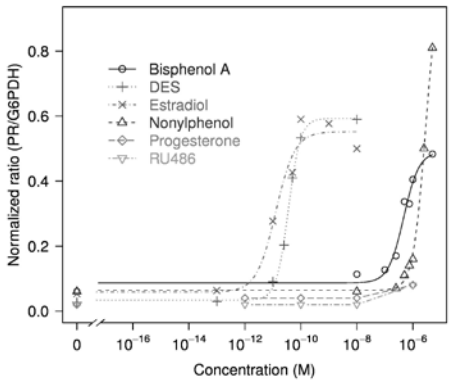


Figure 6. Effects of reference chemicals on PR mRNA levels in Ishikawa cells. 17ß-estradiol, DES bisphenol A, nonylphenol, progesterone and RU486 were tested over 24 h of culture after 3 d pre-culture. Results are given as normalised ratios. EC_{50} were 3 x 10-11 M (17ß-estradiol) and 1x 10-11 M (DES), 4.7 x 10-7 M (bisphenol A) and 3 x 10-6 M (nonylphenol).

Matts Ollovsson from the Uppsala University, SE, investigated the effects of some putatively endocrine disrupting chemicals (EDCs), hormones, and other hormonally active substances on freshly isolated and *in vitro* grown HEEC via qRT-PCR in order to better mimic the *in vivo* situation. HEEC were also co-cultured with human endometrial stromal cells that communicate with the HEEC in human tissue. Mifepristone, tamoxifen and cadmium chloride all affected the expression of genes of importance for the regulation of angiogenic activity in the HEEC at concentrations that did not affect HEEC viability and proliferation. Genes

relevant for the control of angiogenesis thus seem to constitute reasonable and useful toxicological endpoints. This *in vitro* model might serve as one of the tests in a panel that can discriminate between chemicals with or without adverse effects on human reproductive functions. However, further studies are needed and we will focus on the more promising co-culture systems. (For publications in WP II.1 see references 18-22.)

WP II.2 Placental toxicity

Mammalian placentation is a critical process potentially sensitive to adverse effects of chemicals. In order to identify critical targets in the process of placentation at early stages of pregnancy, the functions of selected human cell types (trophoblasts and/or pericytes) were assessed. In addition, a new system using the BeWo trophoblast line allows a higher throughput of chemicals.

Luana Paulesu and her group at the Universitá degli Studi di Siena, IT, exposed cells of the trophoblast lines BeWo and HTR8/Svneo to test chemicals in a wide range of concentrations. By assaying the release of β-hCG, an essential hormone for pregnancy, the endocrine activity of BeWo cells could be determined. As HTR8/Svneo cells are representative of the extravillous trophoblast, these cells do not release detectable amounts of β-hCG precluding a comparison between the effect of chemicals on cytotoxicity and that on the loss of endocrine activity. In both cell types, estrogenic test compounds induced a concentration-dependent decrease in cell viability. For the BeWo cells the LC_{50}/IC_{50} ratio was derived as a specificity index, ranking test chemicals according to their potency to impair hormone secretion.

In vitro tests using primary cultures of chorionic villous explants (obtained from first trimester human placenta) represent an elective model for functional studies in human placenta. With this model it is possible to monitor trophoblast differentiation, both into syncytio-trophoblast (ST) and extravillous trophoblast (EVT). As for differentiation of trophoblast to the ST, the group examined the secretion of β-hCG. The expression level of caspase-3 served as a suitable marker of trophoblast differentiation and its apoptotic shedding. As for the differentiation of trophoblast toward the invasive trophoblast, the release of metalloproteases MMP-2 and MMP-9 were assessed. Results reported in Table 2 show that most of the estrogenic chemicals tested were significantly increasing secretion of β-hCG and MMP-2. Evidence of cell apoptosis in explant tissues exposed to p-NP, BPA, RES and E2 but not to ATZ and DES was also revealed.

Another aim of the project was to evaluate the use of a human placenta derived mesenchymal stem cell-like *in vitro* model (pericytes). Overall, the data generated by Lennart Dencker at the Uppsala University, SE, on model toxicants (cadmium chloride, valproic acid and analogues, HDAC inhibitors) do not support the role of placental pericyte cells as a general *in vitro* screening system for reproductive toxicity. The biological

Table 2. Variation in β-hCG, MMP-2 and MMP-9 secretion by human chorionic villous explants after exposure for 48 hrs to chemicals tested at the concentrations selected.

Chemical	Concentration	β-hCG	MMP2	MMP9
Atrazine (ATZ)	1 µM	⇔	⇔	⇔
Bisphenol A (BPA)	1 nM	⇑	⇔	⇩
Diethylstilbestrol (DES)	1 nM	⇔	⇑	⇔
Para-Nonylphenol (p-NP)	1 nM	⇑	⇑	⇩
Resveratrol (RES)	1 µM	⇑	⇑	⇓
17β-Estradiol (E2)	1 nM	⇑	⇔	⇔

Significant difference: (⇑ = increase) (⇓ = decrease), no significant difference: (⇩ = decrease) (⇔ = no change), vs control (vehicle exposed) cultures.

variation between human placentas and the cell isolation methodology in itself was not compatible with an easy-to-use *in vitro* system. However, the cell system may be instrumental in the study of specific aspects such as angiogenesis during development.

WP II.2 also concentrated on placental transfer of chemicals as part of the exposure element in risk assessment related to developmental fetotoxicity. Comparison of transplacental transfer, especially using the reference compound antipyrine, has been carried out between two laboratories, one at the University of Copenhagen, DK (Lisbeth Knudsen), the other at the University of Kuopio, FI (Kirsi Vähäkangas), using the human placental perfusion system (Figure 7).

Both antipyrine transfer and the leak from fetal to maternal circulation were used as criteria for successful perfusions. Antipyrine results were very similar between the laboratories, and the same applies largely to the heterocyclic amines PhIP and IQ. However, the clearest differences were noted in the kinetics of the lipophilic compound benzo(a)pyrene, which e.g., binds to the tubing of the system. More studies are needed to clarify

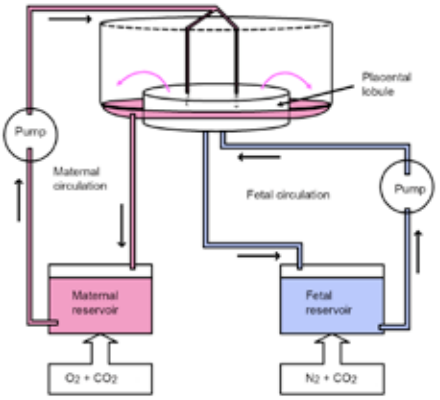


Figure 7. Schematic drawing of the dual re-circulating human placental perfusion system. The maternal circulation is shown in pink and the fetal in blue.

whether the perfusion model is applicable to highly lipid soluble compounds. In general, the transplacental transfer of the compounds studied was significant. In the experiments by Kirsi Vähäkangas, acrylamide and glycidamide were evidently transported by passive diffusion and their transfer was similar to that of antipyrine. Transfer of PhIP was restricted compared to the transfer of antipyrine. On the other hand, another heterocyclic amine, IQ, seemed to go through the placenta like antipyrine and was thus different from PhIP.

Lisbeth Knudsen analysed the placental passage of compounds with different physicochemical properties (caffeine, benzoic acid, and glyphosate) and compared the results with those on the transport of the these compounds (plus the reference compound antipyrine) through BeWo (b30) cell monolayers. It turned out that both models of placental transport (the BeWo cell line and *ex vivo* placental perfusions) show similar results in ranking the compounds according to their transfer rate. A step-wise approach should therefore be implemented for ideal planning of future research projects. The *in vitro* BeWo cell transport experiments might be employed as an initial screening tool. If the results from BeWo cell experiments are consistent with expectations based on physical and chemical characteristics and comparison to analogous compounds, appropriate data classification from these *in vitro* experiments may be sufficient.

To examine the effects of bisphenol A (BPA) exposure during pregnancy, Lisbeth Knudsen and Luana Paulesi performed studies using the BeWo trophoblast cell line, placental explant cultures, placental perfusions and skin diffusion models, all of human origin. The *in vitro* results with BPA indicate that low, environmentally relevant concentrations of BPA can affect hCG secretion of the early trimester trophoblast and increase apoptotic activity with potential adverse effects on placental development. Furthermore, BPA is transported across the term human placenta, the trophoblast cell monolayer, and the human skin, which strongly implicate the potential for fetal exposure to BPA, with the risk of hormonal and developmental disruption. These test systems, within the realm of human implantation and fetal development, are important elements of reproductive toxicology risk assessment. (See references 23-41 for WP II.2 publications.)

Research Area III:

WP III.1 Early Embryonic Development

Currently, one of the probably most promising cell culture systems for the detection of the embryotoxic hazard of chemicals is based on embryonic stem cells. The Embryonic Stem Cell Test (EST) is currently the only test system that is based on a mammalian cell line and has successfully been validated. However, the test detects predominantly chemical effects on cardiac cell differentiation. Effects on other major target tissues such as the developing nervous system or the developing skeletal system might not be reliably predicted. The partners focused on the further development of the EST in order to identify adverse effects on other major target tissues (neural and skeletal tissues).

Another objective was the enlargement of the database for the validated endpoints, which was performed both by the groups of Horst Spielmann and Andrea Seiler at ZEBET, Berlin, DE, and Margino Steemans at Johnson & Johnson, Beerse, BE. From the results of the study of the effects obtained with 13 new test agents, it became clear that the validated EST prediction model failed to predict the activity of the new test compounds correctly. One reason for the poor prediction might be that the ReProTect list of chemicals for developmental toxicity testing uses four teratogen categories (non, mild, moderate, strong), in contrast to the classification used for the validated EST (non, weak, strong). The analysis also showed the limitations of the test to discriminate weak- from non-embryotoxicants. It is a success of ReProTect to better define the applicability domain and the limitations of the EST.

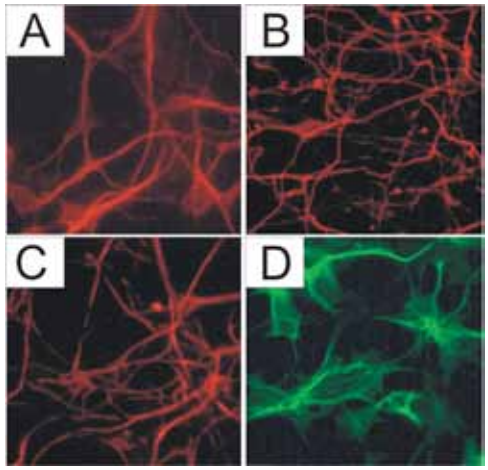


Figure 8. Immunofluorescence staining of neural cells. Neural cells were stained with antibodies for neuron marker proteins MAP2 (A), b-III tubulin (B), a-internexin (C) and for the astrocyte marker protein GFAP (D).

For identification of new molecular markers for embryotoxicity, a differential proteome analysis was applied by the group of André Schrattenholz, ProteoSys AG, Mainz, DE. Protein lysates from chemically-treated and untreated mouse embryonic stem cells (mESCs) differentiated into cardiomyocytes and into neural cells, as well as from undifferentiated stem cells, were prepared by the group of Horst Spielmann and Andrea Seiler and transferred to ProteoSys for proteome analysis. ProteoSys finally succeeded in identifying new protein biomarkers, which appear to be suitable to be used for *in vitro* testing for embryotoxicity.

In the validated version of the EST, embryonic stem cells are differentiated into cardiomyocytes. To allow the detection of developmental neurotoxicants, a neural cell differentiation protocol was adapted from literature. The differentiation of the mESCs into neural cells was characterised by analysis of neuron-specific markers (MAP2, a-internexin and b-III tubulin, GFAP) using intracellular flow cytometry and immunofluorescence staining with neuron-specific antibodies to confirm cell-type specificity (Figure 8). Analysis of neuron-specific marker gene expression (MAP2) at day 12 of differentiation by flow cytometry was established as an endpoint to detect developmental neurotoxic effects of chemicals. The neuronal differentiation protocol was successfully transferred to Margino Steemans at the Johnson & Johnson laboratory. mESCs were also successfully differentiated into chondrocytes by the group at ZEBET.

The use of human embryonic stem (hES) cells for embryotoxicity testing could help to avoid difficulties of hazard identification due to inter-species variations. Differentiation of neural precursors was established by Tina Stumman and Susanne Bremer at the ECVAM laboratory at the JRC and characterised by studying the kinetics of neural mRNA marker expression. The result showed a sequential expression of markers, which was paralleled by the occurrence of rosette structures present during neural tube formation *in vivo*. Applying the neuronal embryotoxicant methylmercury in a non-cytotoxic concentration during differentiation resulted in the reduction of neural mRNA marker expression indicating the validity of the assay for detection of neuronal embryotoxicity. (For publications in WP III.1 see references 42-45.)

WP III.2 Late Embryonic Development

Rodent postimplantation Whole Embryo Culture (WEC) is the only available *ex vivo* test for organ formation during embryogenesis. It is widely used, both in mechanistic studies and as a screening test for developmental toxicants. Endpoints used in the WEC are a series of well-defined morphological endpoints: all primordiums receive a score dependent on their developmental stage, and all scores added up give the so-called Total Morphological Score (TMS). Besides this score, malformations and size measurements are noted, the latter comprising of yolk sac diameter, head length and crown-rump length (Figure 9).

The objective of the work of Aldert Piersma's group at RIVM, Bilthoven,

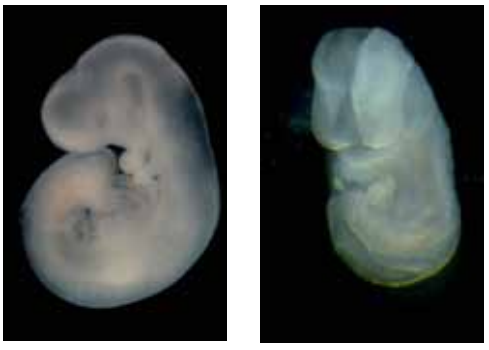


Figure 9. Normal control embryo (l) and exposed malformed embryo with open neural tube (r) after 48 hour culture *in vitro*.

NL, was the introduction of a metabolic incubation system into the WEC that will bio(in)activate chemical compounds to the metabolites that occur within the human body in real life, and are therefore relevant for the induction of biological effects. After testing a number of possible metabolic systems with cyclophosphamide (CPA) activation as the positive control, rat liver microsomes were selected as the metabolic unit. This choice was made on the basis of adequate activation of CPA in combination with the absence of toxic effects of the metabolic incubation mixture in embryo culture. The selection of 8 additional test compounds was then guided by the ReProTect Metabolic Activation Group under the chairmanship of Heinz Nau, University of Hannover, DE. CPA and 2-acetaminofluorene were successfully activated in the preincubation system, while methoxyethanol and retinol appeared not to be activated. This is most probably due to the fact that the dehydrogenases which are necessary for the metabolic activation of these compounds are cytoplasmic enzymes which are not present in microsomes. This study therefore confirms the limitations of using microsomal metabolic systems. Their metabolic enzyme profile is not complete as a result of which some compounds will be activated and other will not. The prospect of this method as a general screening system for embryotoxants that need bioactivation is therefore limited. (For publications in RA III.2 see reference 46.)

Research Area IV: Cross-cutting Technologies

WP IV.1 Sensor Technologies

The applicability of a microelectrode array for the study of developmental cardiotoxicity has been investigated at ECVAM, JRC, Ispra. A preliminary protocol on the assay has been developed and challenged with selected test chemicals. Unfortunately, the test demonstrated high variability of its readout. For this reason the task was stopped at an early timepoint.

WP IV.2 (Q)SARs

It is a challenging task to develop broad-range (Q)SAR models in reproductive and developmental toxicity due to the several, and still somewhat unclear, mechanisms and pathways involved. Nevertheless, (Q) SARs may be efficiently developed to provide an important contribution to the part of hazard characterisation concerned with kinetics and bioavailability. In the (Q_SAR modelling of barrier penetration, it has generally been assumed that membrane penetration occurs by passive diffusion across lipid bilayers. Therefore, most existing (Q)SAR models are applicable to chemicals that cross barriers by passive diffusion rather than by specific forms of transport. Mark Cronin and Mark Hewitt (Liverpool John Moores University, UK) have worked on the development of three (Q)SAR models: a model for blood-testis barrier, a model for placental transfer, and a model for P-glycoprotein-mediated transport. Despite the scarcity

of suitable data to allow modeling of the human blood-testis barrier, an attempt was made to model the largest dataset found in literature (consisting of only 10 compounds). Unfortunately, the resulting model showed poor statistical fit and prediction.

In order to develop (Q)SAR models to predict the transport of a substance across the human blood-placental barrier, the group used human placental perfusion data in the form of clearance or transfer indices (CI or TI), each using antipyrine as a reference compound. These data were collated and (Q)SAR models derived. The study has shown placental transfer to be dependent upon a number of factors—hydrogen-bonding and hydrophobicity being the most influential.

Active transport processes of placental transfer were taken into account by the generation of a separate (Q)SAR model for the best-studied transporter of the placenta, P-glycoprotein (P-gp). Five data sets were collected and multiple techniques were implemented to obtain the best possible model. No improvement could be achieved, however, as compared to previous studies, which is likely a result of P-gp possessing multiple binding sites and having an extremely wide substrate specificity. Pharmacophore modelling was also unsuccessful, which is a result of the vast structural variability between substrates. However, the models still proved useful in predicting which compounds may be P-gp substrates, and hence yielded poor placental transfer predictions.

An *in silico* liver metabolic simulator was developed by Ovanes Mekenyan, Bourgas "Prof. As. Zlatarov" University, BG. Mammalian *in vivo* liver metabolism data were mined from multiple bioassays to expand as much as possible the simulator applicability domain. The rational combining of *in vivo* and *in vitro* data is also driven by the fact that most *in vitro* tests (such as S9 fraction and primary hepatocytes, as well as liver slice bioassays) provide phase I and phase II metabolism, and the observed metabolic profile is similar to *in vivo* metabolism. Documented maps for 203 chemicals (mostly rat *in vivo* data) were collected from the literature and internet. These data were then used to upgrade the liver metabolic simulator developed at LMC in terms of: (1) expanding the simulator applicability domain, and (2) improving the simulator.

The metabolic simulator was then combined with a (Q)SAR model for binding affinity of chemicals to the human estrogen receptor alpha (hERα). The hERα binding affinities were modelled using a training set of 645 chemicals. The ultimate model was organised as a battery of (Q)SARs associated with interaction mechanism and potency bins categories. The (Q)SAR model and the metabolic simulator were combined in the so-called TIMES modelling platform. When a new chemical is submitted to the system, it undergoes simulated metabolism, such that part of the generated metabolites are detoxified by phase II reactions so interrupting the metabolis while other metabolites are filtered by the (Q)SAR model to predict

their binding potency to hERα. To assess the model applicability the simulator was used to predict expected metabolites for more than 150 chemicals. The developed two-step model can be used as a source for supporting information in a weight-of-evidence approach.

A general problem inherent in all *in vitro* test systems is that the pharmaco/toxicokinetics of test chemicals are not portrayed. So far, *in vitro* tests have been mostly used for hazard identification (presence or absence of a toxic response), but not for the establishment of dose-response relationships, which would be a prerequisite for implementation of such assays into new integrated strategies for risk assessment. Ideally, *in vitro* effect levels are first determined in a relevant cell- or tissue-specific *in vitro* assay and then used by an *in silico* approach to predict target organ concentrations and whole organism toxicity effect levels. Once this has been achieved, physiologically-based pharmacokinetic (PBPK) modelling approaches can be used to extrapolate the calculated plasma levels to *in vivo* effect levels predicted to occur following different routes of exposure. This approach has been applied by Miriam Verwei and colleagues at TNO, Zeist, NL, to a series of embryotoxic compounds tested in the EST, and the results of the study were quite promising as they demonstrated a fairly good correlation between effect levels predicted *in silico*-predicted and *in vivo* levels reported in the literature for six out of seven tested. (For publications in RA IV.2 see references 47-53.)

WP IV.3 Metabolic activation

One of the major problems with all *in vitro* assays, including the EST, is the lack of a suitable metabolic activation system. The group of Heinz Nau from the University of Hannover, DE, has investigated various systems to complement the EST with a bioactivation system. The training set of chemicals contains the well-known proteratogens valpromide (VPD) and cyclophosphamide (CPA). Initial experiments using the so-called S9 mix together with the embryonic stem cells failed because of toxicity of the liver homogenate. Further experiments using freshly isolated rat hepatocytes turned out to be the most promising system to combine with the EST. Due to the "hanging drop" culture of ES cells during the first three days of the differentiation assay, a co-cultivation of hepatocytes is very difficult to perform in the EST. Thus, a pre-incubation step—combining the hepatocytes with the test compounds—was introduced, after which the supernatant containing the activated metabolites was added to the EST cultures (Figure 10). This system worked well with CPA (Figure 11), but failed with VPD, most likely because the compound was not metabolised by the hepatocytes to the active metabolite valproic acid, unlike the *in vivo* situation. The results show that the combination of the EST with a metabolic activation system using a pre-incubation step provides a valuable advancement, but has clearly its limitations. To avoid misclassifications of test substances caused by inter-species variations (e.g., difference in metabolic activation of

VPD between mice and humans), further research on the development of a human cell-based test-system would be advantageous. (For publications in RA IV, WP II see references 54-57.)

WP IV.4 Array technologies

Within this work package, the group of Alberto Mantovani (Istituto Superiore di Sanità, Rome, IT) has established a strategy to detect the transcriptomic signature of endocrine disrupting chemicals (EDCs) interfering with androgen-receptor (AR) pathways. LNCaP cells were used to assess differentially-modulated genes of various androgens and anti-androgens in comparison with vehicle-treated controls. Data expression analysis of 1651 selected genes showed that it is possible to identify specific molecular signatures distinguishing androgenic and anti-androgenic chemicals that act through AR-mediated pathways.

In parallel, the prostate-specific antigen (PSA) secretion—as an established biochemical marker in clinical medicine—was used as a functional marker for phenotypic anchoring of the transcriptome analysis. The PSA performance for phenotypic anchoring was satisfactory based on the overlap with toxicogenomic data.

The PSA secretion assay has been preliminarily developed as an additional reproductive endpoint targeted by endocrine disrupter action, i.e., prostate function. The PSA secretion study has been conducted in parallel, as a side

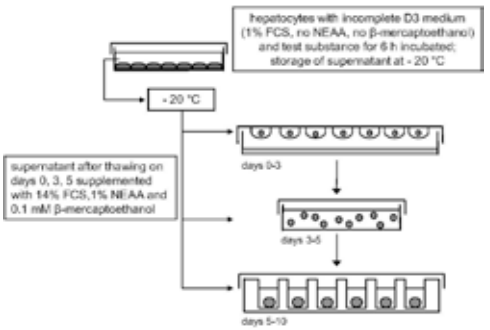
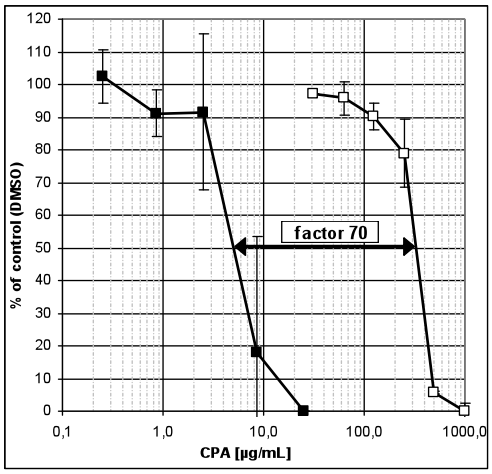


Figure 10. Combination of hepatocytes with the EST.

Figure 11. Comparison of CPA-effects without and after 6 hrs of incubation with murine hepatocytes on differentiation of D3 cells. Data represent means± SD. Number of experiments using non-activated CPA: n=3. Number of experiments with metabolically activated CPA: 25 µg CPA/ml: n=3; 0.3, 0.9, 8.5 µg CPA/ml: n=4; 2.6 µg CPA/ml: n=8.
■ = D3 differentiation with preincubated CPA,
□ = D3 differentiation with non-incubated CPA.



study, within the frame of the ReProTect feasibility study. The proposed assay is time- and cost-effective, since both endpoints (cell viability by MTS assay and the prostate-specific PSA secretion) are performed on the same microtiter-grown cell cultures and can be measured at the same time at the end of treatments. Our results indicated that PSA secretion is a sensitive marker able to discriminate compounds known to affect prostate physiology *in vivo*. In particular, the integrated approach allowed: (i) identificationof a putative endocrine disrupter (the herbicide glufosinate ammonium), whose role as AR-interfering chemical has yet been characterised; (ii) detection, by comparison to linuron and di-n-butyl phthalate, a feasibility-study anti-androgen (vinclozolin), and (iii) pinpointing of the estrogen-mimicking compound BPA. Additional chemicals have to be tested to explore the potential of the assay to properly detect prostate-mediated action on male reproduction. (For publications in WP IV.4 see references 58-65.)

WP IV.5 Receptor assays

Hormonal activity of chemicals is a matter of concern and has been linked to disruption of the endocrine system in wildlife, and may possibly also be relevant for humans and their reproductive function. WP IV.5 has dealt with the respective hormone receptor interactions. Despite more than two decades of research in the field, neither a validated recombinant androgen receptor (AR) or estrogen receptor (ER)-binding assay, nor a validated cell-based androgen- or estrogen-sensitive transactivation assay, is available. Within the ReProTect project, a rat recombinant AR-binding assay (ARBA) and a human recombinant full-length ERα-binding assay (ERBA) have been developed, as well as highly sensitive

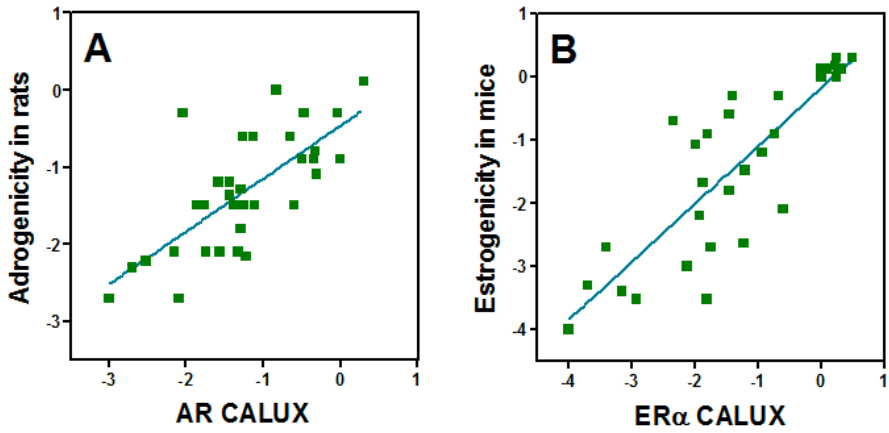


Figure 12. Androgenicity and estrogenicity of chemicals can be predicted with AR CALUX and ER CALUX reporter gene assays.

cell lines measuring the transactivation of androgens (AR CALUX, PALM) and estrogens (ER CALUX, MELN).

To test chemicals on their estrogenic and androgenic activities, Bart van der Burg and his group, Biodetection Systems, Amsterdam, NL, established and prevalidated the AR CALUX and the ERα CALUX androgen and estrogen receptor assays, which show good correlations with *in vivo* assays determining specific hormonal endpoints (Figure 12).

The *in vitro* CALUX methods have discrete advantages that go beyond reduced speed, cost, and animal use, such as the possibility to use human cells and receptors. These may reduce the correlation with the animal model, but enhance the predictive value of the *in vitro* model. Therefore, the expected purpose of the *in vitro* receptor assays is to be at least at the level of inclusion as part of a hierarchical approach in the context of regulatory guidelines, and even for full replacement of *in vivo* methods.

Internal validation and protocol optimisation has been carried out for ERα and AR CALUX at the lead laboratory in Amsterdam. A novel cell clone of the ERα CALUX cell line proved to be a very suitable and reliable predictor of estrogenicity of chemicals, and was able to readily rank a range of chemicals on the basis of their EC_{50} values. This clone was transferred successfully to two different labs. The established AR CALUX reporter gene assay turned out to be a robust and highly selective method to test androgenic and anti-androgenic activity of compounds

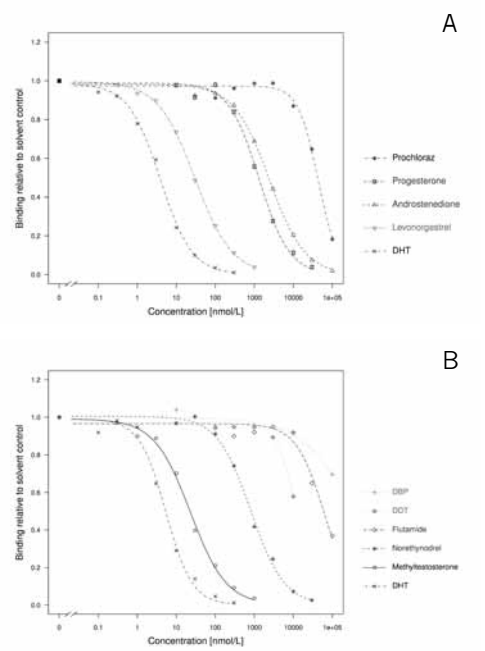


Figure 13. Competitive binding to the rat recombinant androgen receptor of reference compounds dihydrotestosterone (DHT) and androstenedione and of levonorgestrel, progesterone and prochloraz (A). Competitive binding to the rat recombinant androgen receptor of reference compound DHT, 17α-methyltestosterone, norethynodrel, o, p'-DDT, flutamide and dibutylphthalate (B). Representative displacement curves are shown. For the sake of clarity, only means and curve fits are given.

in vitro. The test was transferred to the laboratory of Hilda Witters at The Flemish Institute for Technological Research (VITO), Mol, BE, leading to comparable test results with a panel of androgen receptor agonists and antagonists, demonstrating low between-lab variability. The assay was able to readily rank a range of chemicals on the basis of their EC_{50} values, which

correspond well with known potencies in other assays.

Alexius Freyberger and Hans-Jürgen Ahr at Bayer Schering Pharma AG, (BSP) Wuppertal, DE, developed two receptor-binding assays named ARBA and ERBA. In both assays, the properties of reference and test compounds were well characterised, in terms of relative ranking of binding affinities (Figure 13). The obtained data demonstrate that the assays reliably ranked compounds with strong, weak, and no/marginal affinity for the AR or ER with high accuracy.

Furthermore, the PALM assay, a cell-based AR transactivation assay, was developed at BSP. The assay discriminates receptor agonists from antagonists. It correctly detected the (anti)androgenic properties of reference and test compounds. The PALM assay was also successfully established at a second laboratory, VITO, BE, and between-laboratory comparison of results obtained by VITO and BSP showed only minor differences. Accordingly, the assay has proven to be transferable, robust, and reproducible.

A second cell-based reporter assay for detection of estrogenic activity of test compounds was developed by Hilda Witters and her group at VITO, who established an ER transcriptional assay based on MELN cells (MELN assay), which is accompanied by a cytotoxicity assay with the same cells on one test plate. The cytotoxicity test is based on the measurement of LDH leakage using a fluorescence assay, with the exposed cells remaining available for the measurement of luciferase.

The MELN assay was successfully transferred to the BSP laboratory as a second lab, applying an extended list of ER agonists and antagonists. Evaluation of a data set obtained from a pre-screen with chemicals with known mode of action demonstrated good comparability between the labs, proving the assay to be transferable, robust and reproducible (Figure 14).

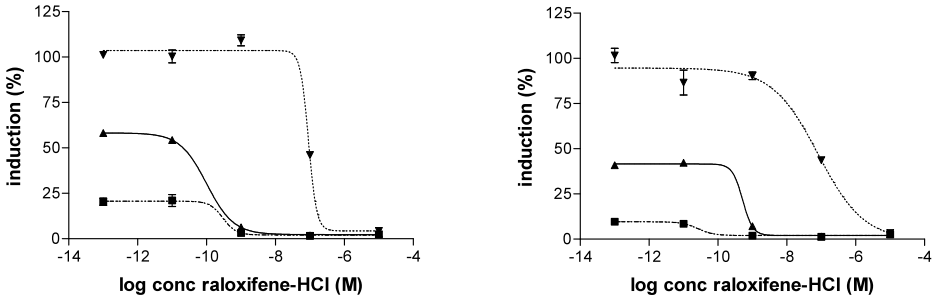


Figure 14. Graphs of prescreen results at VITO and BSP for 1 test chemical (TC) raloxifene-HCl, evaluated in 3 different test modes (n TC; p TC + EC50 E2, q TC + 1000xEC50 E2). The curves represent mean values with SD (n=3) and fitting was made by GraphPad software.

The group of Alois Jungbauer, University of Agricultural Sciences, Vienna, AT, aimed to develop a transactivation system using a yeast triple mutant expressing the ERα, ERβ, and AhR receptor to test for effects mediated by xenobiotics specifically on potentially heterodimerised receptors. In the end, only the AR receptor and a corresponding reporter construct was successfully transfected, while a functional triple mutant could not be established.

The PALM, MELN, and CALUX methods have been selected by the ECVAM ED Task Force as tests that have the potential to be used to reduce the amount of *in vivo* tests for androgenic (AR CALUX and PALM) and estrogenic (ERα CALUX and MELN) compounds. To determine the suitability of the tests for this purpose, a full ECVAM/OECD-headed validation is envisaged. (For publications in WP IV.5 see references 66-76.)

WP IV.6 Stem cell-based reporter assay

The group of the ReProTect coordinator at the University of Tübingen, DE, started in the forth year of ReProTect, a project within Work area IV. The work package aimed to detect effects of potential embryotoxicants on signalling pathways known to be relevant for early embryonic development. The process of embryonic development is stringently regulated by several signalling pathways that are evolutionary conserved from the fruit fly to humans (Figure 15). To detect chemically-induced alterations in the canonical Wnt/β-catenin signalling pathway, a stem cell-based reporter assay was developed.

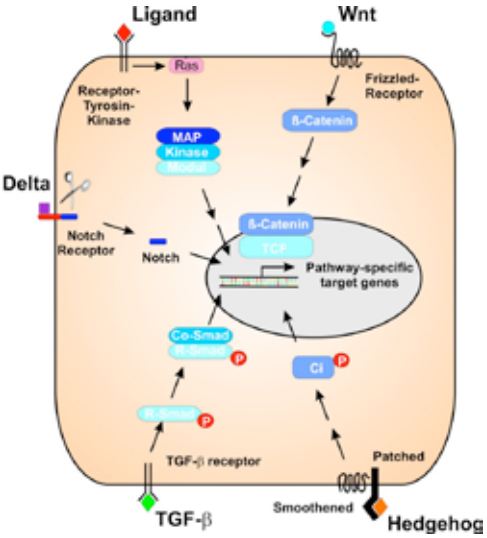


Figure 15. Five signaling pathways that govern early embryonic development.

The so-called ReProGlo assay simultanously determines cell viability and luciferase reporter activity in a high-throughput 96-well microtiter format. A clone of mouse embryonic stem (mES) cells stably expressing the SuperTopFlash reporter was established. This allows Wnt pathway activity determinations in undifferentiated mES cells and their differentiated descendants. Several test chemicals were analysed in the new assay system. Known embryotoxicants such as retinoic acid and lithium chloride induced concentration-dependent increases in reporter activity. The potency of valproic acid and a series of structural analogs to activate the Wnt pathway correlated well with their reported teratogenic activity in the mouse. Cyclophosphamide was active after metabolic activation by hepatocytes. The new test may help to predict

embryotoxic potential of chemicals. Surely, more test chemicals must be applied to address the applicability domain of the ReProGlo assay. The initial results are, however, promising and the test has the advantage of being easy to perform and cost-effective. The integration of ES cell clones expressing reporters for additional pathways of importance for embryonic development may improve the predictivity of the assay. (For publications in WP IV.6 see reference 77.)

WP V-VII Management Activities

The management of the project (WP V) was achieved through close collaboration between the coordinator Michael Schwarz, his assistants Susanne Stoppel and Barbara Schenk in Tübingen, DE, the management team Susanne Bremer and Cristian Pellizzer at ECVAM/JRC, IT, and the area leaders Giovanna Lazzari (WP I), Lennart Dencker (WP II), Horst Spielmann (WP III), and Alberto Mantovani (WP IV). The management team received managerial and scientific advice through the Supervisory Board, chaired by Bernward Garthoff.

Visibility to the public and dissemination of results was achieved through the ReProTect webpage (*ReProTect.eu*), organised within WP VI at the Vrije Universiteit Brüssels, BE, which also prepared a flyer and two brochures on ReProTect. The coordinator and other members of the consortium have given several overview oral presentations on the project and its results, e.g., during the EPAA Conference in Brussels in 2008, the CHMP Safety Working Party Meeting of the EMEA in London in 2009, the 7th World Congress on Alternatives and Animal Use in the Life Sciences in Rome in 2009, the EUROTOX Congress in Dresden in 2009, the 5th Chairs Meeting at DG RTD in Brussels in 2009, and the annual ecopa conference in Brussels in 2009, just to name some.

Finally, a survey on reproductive toxicity tests already used by industry was compiled by the *In Vitro* Testing Industrial Platform (IVTIP) in WP VII.

WP VIII The ReProTect Feasibility Study

In the final year of the project, a ring trial, termed the "Feasibility Study", was

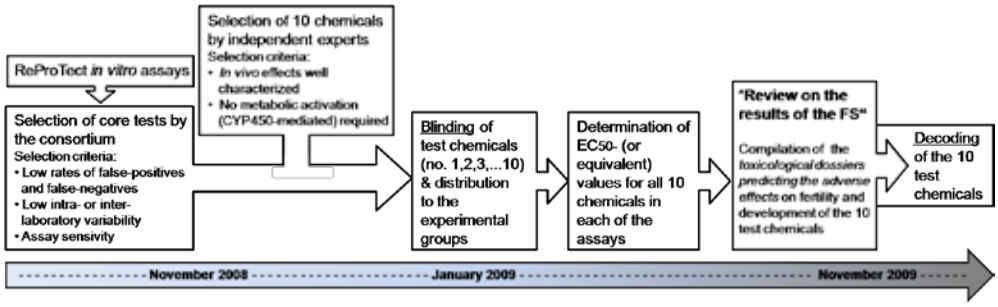


Figure 16. Schematic outline of the ReProTect Feasibility study.

conducted in order to challenge the *in vitro* tests of ReProTect. At a workshop in November 2008, the most advanced tests were selected based on their performance quality and optimisation. A group of independent experts decided on a set of 10 test chemicals, which were sent to the participants in the Feasibility Study and applied to their test in a blinded fashion. The results were collected and evaluated by an independent statistician sponsored by ECVAM. Finally, the outcome of the study was discussed by all participants of the study at a second workshop organised in November 2009. Based on the *in vitro* effects produced by the 10 chemicals across the various tests of the battery, their possible adverse effects on the reproductive system in the whole animal were predicted before decoding the chemicals. The entire setup of the study is schematically outlined in Figure 16.

The outcome of the study revealed that this preliminary test battery was able to correctly predict most of the *in vivo* effects on fertility and embryonic development, while only few predictions were incorrect for explainable reasons (Figure 17).

The ReProTect Feasibility Study has constituted a paradigm of alternative testing batteries and will guide future approaches in this field. Its success is a major step towards reduction and replacement of animal experiments, and portrays the main achievements of the EU project.

	Chemical	Female fertility	Male fertility	Developmental toxicity
1	Methyl acetoacetate	√	√	√
2	Glufosinate ammonium	√	√	√
3	Methoxyacetic acid	X	X	√
4	Sodium chloride	√	√	√
5	Cadmium chloride	√	√	√
6	Carbendazim	√	√	√
7	Nitrofen	√	X	√
8	Nitrobenzene	√	X	√
9	Vinclozolin	√	√	√
10	Bisphenol A	(√)	(√)	(√)

Figure 17. Summary of the outcome of the ReProTect Feasibility study. Green: negative control compound; red: compound produces adverse effect *in vivo*.
√: effect correctly predicted; X: effect not correctly predicted; (√): effect in the animal strongly dependent on route of application; the effect is correctly predicted when compared to results from non-oral application studies.

Summary & Goal

The ReProTect project was aimed at exploring the future role of alternative, mostly *in vitro,* tests in testing strategies aimed at (i) guaranteeing high safety levels, (ii) reduction in animal experimentation, and (iii) minimisation of costs for screening for reproductive toxicity of test agents. Most of the assay systems that were developed in ReProTect are still at a research and development stage. Validation of existing tests was not an aim of the project. However, some of the tests reached the status to fulfill ECVAM's criteria for entering into prevalidation, which was the original aim of ReProTect and can now be officially submitted to ECVAM to be considered for formal validation.

To our knowledge, a study like the Feasibility Study conducted at the end of the ReProTect project has not been carried out before. It is remarkable that the battery approach of this study was able to identify the toxicologically relevant properties of the 10 test compounds with relatively high accuracy, even though a reliable specific test for male fertility was not available. We anticipate that the applied approach may be very valuable in a tiered strategy for reproductive toxicity testing, guiding future studies in the field.

The ReProTect consortium hopes that its work will help to pave the way for a future with less animal experimentation, but is well aware of the stony path to this ultimate goal.

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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 (finished)
Duration:	42 months

Website: http://er-projects.gf.liu.se/~vitrocellomics

Objectives

The objective of the project was to establish stable cell lines that reliably reflect human hepatic properties through 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 could be used by the pharmaceutical industry to replace the use of 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 the 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.

Studies of metabolism and pharmacokinetic properties have become a key activity in the early drug discovery screening programmes. This is mainly driven by the fact that as many as 40% of new chemical entities were recognised to fail in the late clinical phases because of pharmacokinetic problems. Moreover, adverse drug reactions, most of which are pharmacokinetic-based, are the 4th to 6th leading cause of death in hospitalised patients in the US. Therefore, there is an urgent need for *in vitro* tools to predict pharmacokinetics and possible toxic reactions of new compounds at an early stage in drug discovery to be able to select high-quality compounds that could be developed into drugs that are safe and easy to administer to the body. Thus, the pharmaceutical companies have made major investments to screen for relevant metabolic properties early in

the drug discovery process. A major part of current human-related *in vitro* methods is based on fractionated tissue of human origin (usually waste material from operations), primary cells, expressed enzymes, hepatoma cell lines, etc. However, a major problem is still the poor predictive power in the available *in vitro* tools. Today, the pharmaceutical industry still relies heavily on animal models and allometric scaling to predict human pharmacokinetics. Reliable *in vitro* cell models would 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. *In vitro* cell models that could reliably predict human metabolism and disposition would markedly reduce the need for animal experimentation for this purpose.

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 preventing weak lead candidates from entering clinical phases through the application of 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).

Experimental Design

The means to accomplish the objectives of the VITROCELLOMICS project were, in addition to development of new stable hESC-derived hepatocytes, (1) 3D hepatic cell culture and co-culture methods, (2) microcultivation monitoring systems for *in vitro* screening, (3) genomic and metabolomic characterisation, and (4) a multi-microbioreactor platform for high-throughput screening of drug candidates.

The experimental design of the project involved improvement of culturing procedures for directing hESC differentiation towards a mature hepatocyte phenotype, and expansion and handling of the de-

rived hepatocytes. This work also included identification of hepatocyte markers and a thorough characterisation of the phenotype of hepatocyte cells after undergoing the new differentiation protocols.

3D bioreactor culture procedures were developed and adapted in order to further improve hepatic functionality over longer time spans, and miniature bioreactors were developed to increase capacity for high-throughput screening.

Methods to assess metabolism of drugs, clearance and induction of liver enzymes (CYPs) were evaluated and adapted (Table 1).

Established assays for toxicity were evaluated with reference hepatocytes for comparisons with the hESC-derived hepatocytes, and new biosensor technologies were evaluated for use in toxicity screening. The comparative studies of hepatocytes derived from hESC with established *in vitro* models and hepatocyte reference cell types were carried out in order to validate the new models and methods. The biosensor technologies include the optical sensing of oxygen for the measurement of respiration. Respiration can be correlated to viability of the cells (Noor et al., 2009; Beckers et al., 2009). This method is used for non-invasive dynamic monitoring of effects of tested drugs. The method is correlated with commonly used toxicity assays (Noor et al., 2009). An example of another optical biosensing method developed in the project with potential for prevalidation was multi-wavelength fluorescence spectroscopy (Fritzsche et al., 2009).

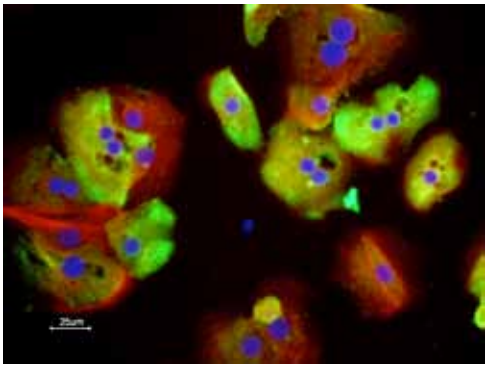

Figure 1. Immunocytochemical staining of hESC-derived hepatocyte-like cells. Positive staining for metabolising enzyme CYP3A4 and liver marker CK18 are shown in green and red colour, respectively. DAPI staining for cell nuclei is shown in blue.

Results

The protocols used to direct differentiation of hESC lines towards mature hepatic phenotype have been continuously modified, refined, and tested. A milestone 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 proj-

Table 1. Summary of pre-validation status of CYP-induction method with HepaRG cells.

Name of the test method	Induction of Cytochrome P450 enzymes in HepaRG cell line applying an "n-in-one approach"
Clinical endpoint	Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4
Cell (line)	HepaRG
Method description	The human hepatoma cell line HepaRG displays hepatocyte-like functions and functionally expresses drug-detoxifying enzymes at relatively high levels, drug transporter proteins and nuclear receptors. In order to assess as much information as possible from one experiment, a n-in-one approach will be followed. After exposure to the test compound at 6 concentrations in triplicates, a cocktail comprising of four selective CYP enzyme substrates, namely phenacetin (CYP1A2), bupropion (CYP2B6), diclofenac (CYP2C9) and midazolam (CYP3A4—not applied in Phase 3T) is added to each well of a 96-well plate. Formation of all specific products, namely acetaminophen (CYP1A2), hydroxybupropion (CYP2B6), 4-hydroxydiclofenac (CYP2C9) and 1-hydroxymidazolam (CYP3A4) is performed after acetonitrile precipitation. For normalisation of enzyme activity to protein content, cells are lysed and protein content is determined by BCA assay. Analytical quantification of metabolites in incubation supernatants is performed by LC/MS-MS.
SOP	Yes
Endpoints	Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4, measured as increase of formation rates of acetaminophen (CYP1A2), hydroxybupropion (CYP2B6), 4-hydroxydiclofenac (CYP2C9) and 1-hydroxymidazolam (CYP3A4
How is a positive result defined?	Fold induction rate >2
How is a positive result expressed?	Fold induction
Applicability	The assay can be applied for the risk assessment of drug-drug interactions of potential new drugs
Positive control	CYP1A2: b-naphthoflavone, CYP2B6, CYP2C9, CYP3A4 rifampicin

Negative control	CYP1A2rifampicin, CYP2B6, CYP2C9, CYP3A4 b-naphthoflavone
Performance	Sensitivity, specificity, accuracy, positive and negative predictive value
Can the test method be used in a regulatory safety context?	Yes/EMEA
Which R would the test method impact?	Replacement/Reduction/Refinement
How can the test be used?	As a stand-alone method

ect. 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 (Figures 3 and 4).

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 was measured in both 96-well Oxoplates and 24-well OxoDishes (Figures 5A and C), which are based on the measurement of luminescence intensity and decay-time, respectively. Both methods are non-invasive and enabled 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 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

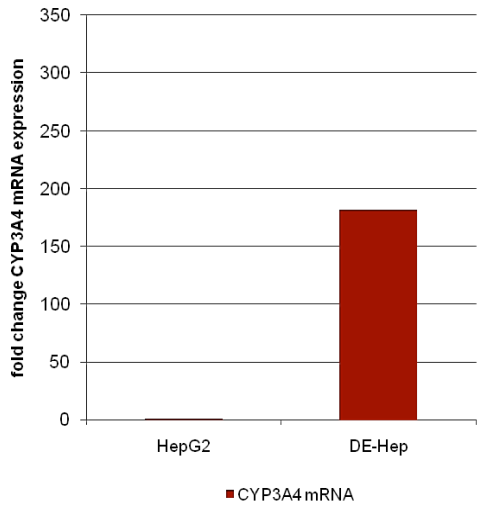

Figure 2. QPCR data for mRNA-expression of CYP3A4 for human cell line HepG2 and hESC-derived hepatocyte–like cells (DE-Hep). Expression level in HepG2 is set to 1.

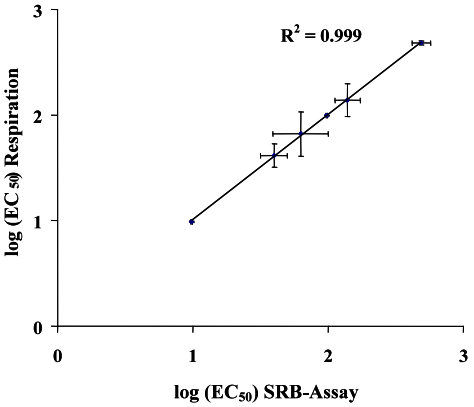

Figure 3. Comparison of EC_{50} values obtained in respiration assay versus those obtained in SRB (protein) assay showing an excellent correlation.

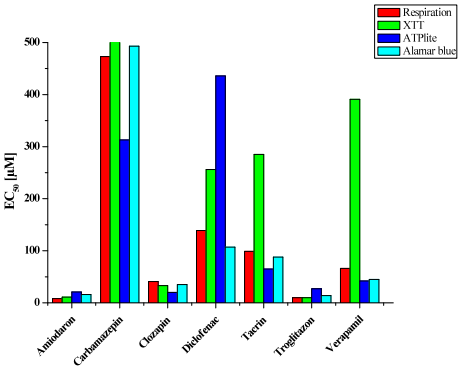

Figure 4. Comparison of EC_{50} values obtained in various cytotoxicity assays and on-line dynamic respiration assay.

Figure 5. SensorDish reader with OxoDish (A). Setup of multiple OxoDishes connected in series in a humidified cell culture incubator (B). A 96-well Oxoplate (C).

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 amiodarone were tested in a range of concentrations for the determination of the EC_{50} values.

Fluxomics. Metabolite balancing and 13C-labelling studies were carried out. Using metabolite balancing, a flux map was established for Hep G2 cells (Figure 6). 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 labelling experiments giving the pentose phosphate pathway activity was included. A simplified model is depicted in Figure 7. Using [1, 2-13C]-glucose, the labelling in lactate was quantified and the mass isotopomer ratios (Figure 8) 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 (Figure 9). 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

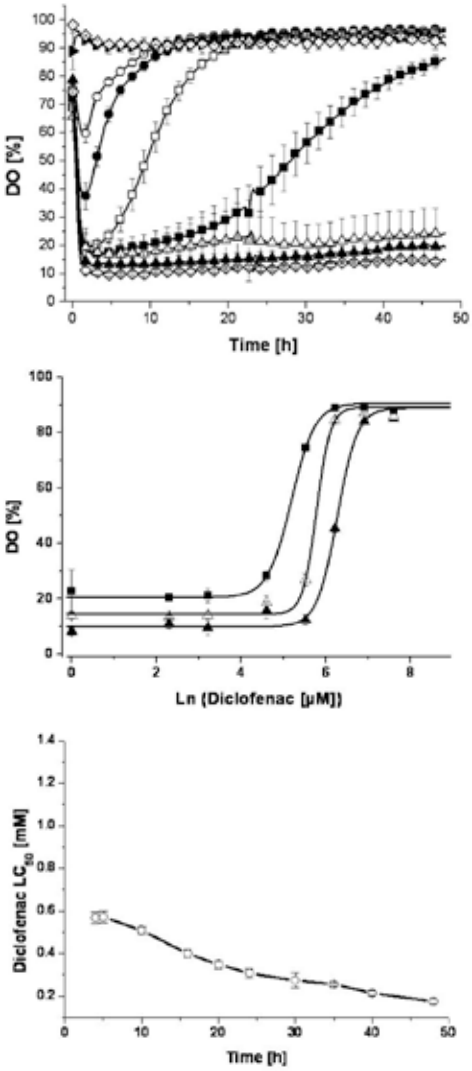

Figure 6. Dissolved oxygen concentration profiles. Diclofenac concentrations a) 0 mM (▲), 0.1 mM (Δ), 0.25 mM (■), 0.5 mM (□), 0.75 mM (●), 1 mM (○) and controls; medium without cells but 1 mM diclofenac (◈), medium without cells with 1% DMSO (►) and cells with medium and 1% DMSO (◊) in PRH; b) Concentration-response curves of PRH calculated at different time points: 10h (▲), 24h (Δ), 48 (■). All measurements were carried out in triplicates. c) Kinetics of toxic effect of diclofenac over time.

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 (Figure 10). The introduction of a down-scaled bioreactor prototype in combination with partial automation of the perfusion periphery enabled studies on hepatocyte metabolism with reduced

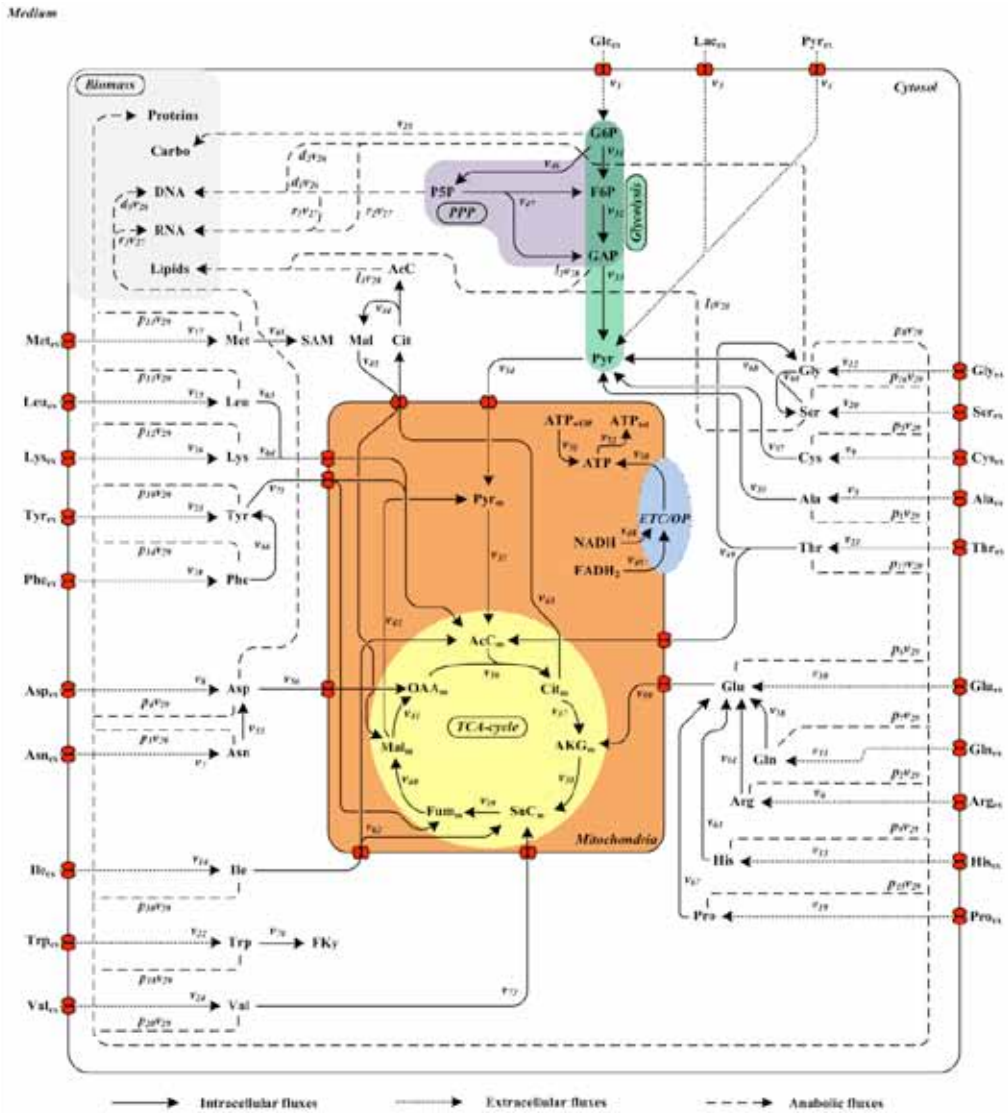

Figure 7. Metabolic network model for the determination of metabolic fluxes in Hep G2 cells.

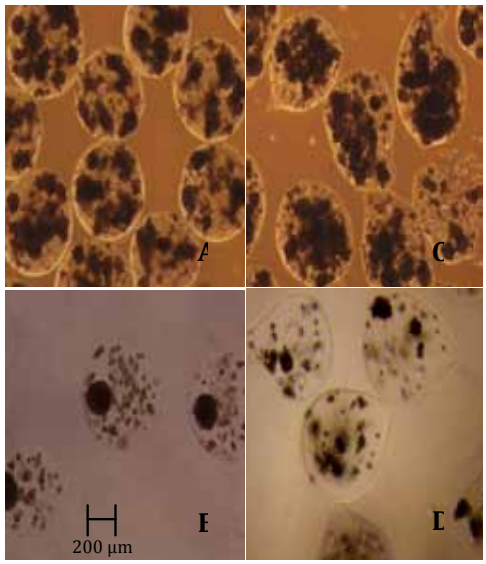

Figure 7B. 3D-entrapped hepatocyte cells.

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 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 prevalidated using HepaRG reference cells provided by BioPredic (Table 1). The structure of the prevalidation study was based of the modular approach proposed by ECVAM (Hartung et al., 2004). The prevalidation study was performed by Pharmacelsus (lead laboratory), IBET, and ECVAM (naïve laboratories).

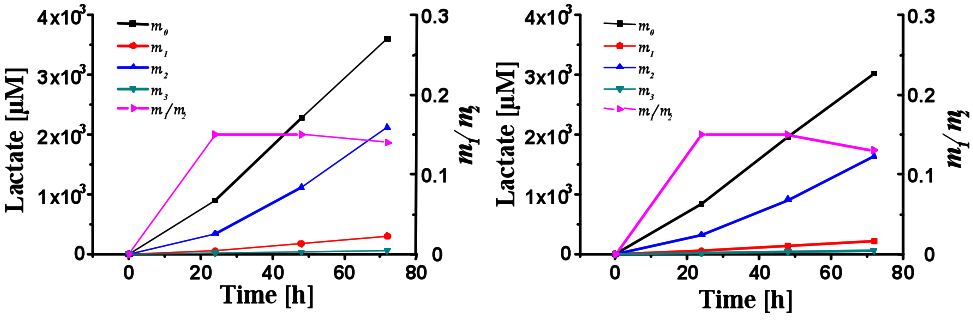

Figure 8. Mass isotopomer concentrations and m1/m2 mass isotopomer ratios of lactate during growth on [1, 2-^{13}C] glucose; a) untreated control; b) treatment with 50µM tacrine.

Figure 9. 3D bioreactor with fully controlled conditions.

The prevalidation consisted of four phases:

1. In the first phase, within-batch reproducibility was tested.
2. In the second phase, between-batch and within-laboratory reproducibility were 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).

The first set of experiments, corresponding with Phases 1 and 2, were performed by Pharmacelsus. Functional enzyme activity assays were performed subsequently to 48-hours of exposure to chemical induc-

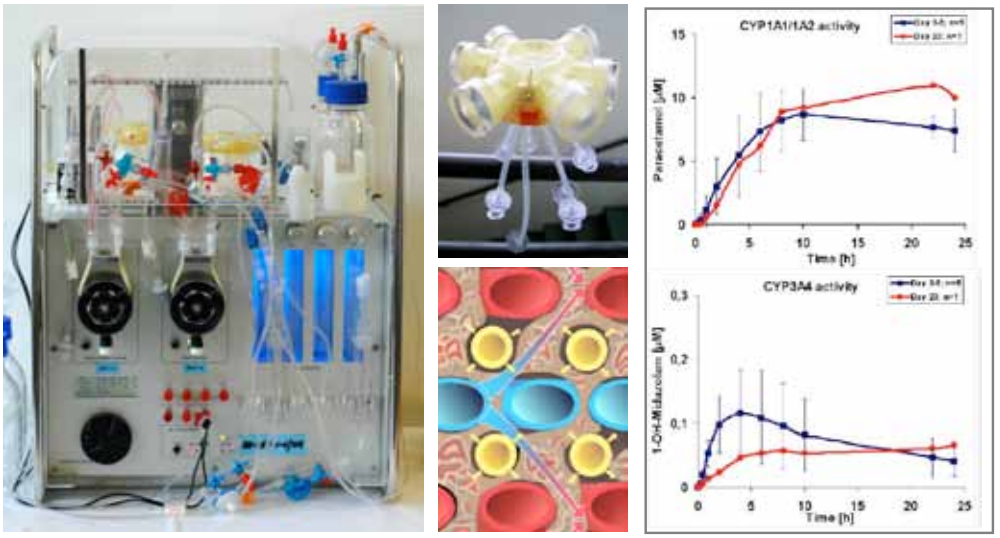

Figure 10. Four-compartment artificial liver bioreactor with three independent capillary bundles for culture medium perfusion (red and blue) and oxygenation (yellow) of the cells located in the extracapillary space. Long-term stability of cytochrome P450 enzyme activities was demonstrated in human hepatocyte bioreactors.

ers, performed by one or two operators at the lead laboratory. Naïve laboratories performed a transferability training assay in order to initiate Phase 3 of the prevalidation study. Furthermore, partners established SOPs for selected assays, guided by partner ECVAM providing the consortium with expertise on test method validation.

Patent Applications Filed / Exploitable Results

- Integration of a Cytochrome P450 induction assay on primary human hepatocytes to the Pharmacelsus portfolio.
- Integration of the (prevalidated) method for Cytochrome P450 induction on the HepaRG cell line to the Pharmacelsus portfolio.
- 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.

Next Steps

Now that the VITROCELLOMICS project is finished, the main remaining action is to make the results available for further studies. In addition to the published scientific reports and reports under submission, an invited review article will be published in *Toxicology In Vitro* that will comprehensively summarises the results. The partners will support **AXLR8** and other actions taken to disseminate the VITROCELLOMICS results.

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MEMTRANS

Membrane transporters: *in vitro* models for the study of their role in drug fate

Contract Number:	LSHB-CT-2006-518246
Project Type:	Specific Targeted Research Project (FP6)
EC Contribution:	€ 1 900 000
Starting Date:	1 April 2006
Duration:	42 months

Website: http://www.acrossbarriers.de/memtrans0.html

Background

The MEMTRANS project focuses on refining and improving *in vitro* methodologies to reduce animal experimentation during the study of the biopharmaceutical and pharmacological properties of drugs with respect to their interaction with transporters, in particular with P-glycoprotein. It could lead to the progression of better drug candidates and therefore it could contribute to making drug discovery and development more successful. The general goal of the project is to optimise and prevalidate existing *in vitro* models for the study of the impact, mechanism, and regulation of drug and xenobiotic efflux transporters. In particular, we wanted to explore the impact of using standardised protocols in reducing the inter-laboratory variability in order to allow the comparison or combination of data coming from different laboratories.

Objectives

The objective of this project was the prevalidation of different *in vitro* cell models of the intestinal membrane to study the relevance of efflux processes mainly mediated by P-glycoprotein. The baseline concept of the project was to use markers of the critical parameters of the cell system (i.e., paracellular permeability, transcellular permeability and transporter expression level) as a weighting factors to translate (or to convert) permeability values obtained in one laboratory to another, allowing the combination

of data from different laboratories and as a method to deal with inter- and intra-laboratory variability, and the first step to achieve this objective is to agree about common Standard Operating Procedures (SOPs) and testing the performance of this standardised methods among the different laboratories.

Experimental Design

1. Selection of a data set of compounds being substrates of Pgp.
2. Selection of three cell lines: Caco-2, MDCK and MDCK-Mdr1.
3. Agreement about the SOPs for culturing and transport experiments.
4. Selection of the markers: LuciferYellow, Metoprolol, and Rhodamine.
5. Evaluation of the markers' permeabilities in the different labs using the SOPs to explore the inter- and intra-laboratory variability.
6. Developing permeability experiments in the three cell lines at different initial concentrations of all the compounds in the different laboratories, simultaneously with the markers' compounds.
7. Comparison of the Peff values in the different laboratories, and statistical analysis of the correlation of the Peff values and the markers' compounds' permeabilities.

Results

Status

All points described in the experimental design have been essentially completed, and the publication of the results is in preparation.

In summary, for the three cell lines assayed, although intra-assay repeatibilities were within acceptable limits, intra-laboratory RSD % values are high in spite of the use of standardised protocols, meaning that the high variability is in some way intrinsic to *in vitro* methods of this kind. On the other hand, this indicates that there are significant differences when results from different days are monitored for a long period of time. This can most probably be due to the different passage number and age of the cells. On the other hand, all results met the qualification criteria; in other words, it was possible to demonstrate the low permeability with either Mannitol or Lucifer yellow, the high permeability with Metoprolol, and P-gp efflux with Rhodamine 123. These results can find use when correlated with the permeation experiments of selected substances to make the intra- or inter-laboratory differences meaningful. Furthermore, experimental data gathered from the selected substances can be normalised using these results as a function of cell performance to minimise the difference between laboratories.

Problems	Solution(s)
The main problems that arose during the project development were related to the analytical procedures, and to the sample transference between laboratories.	It is advisable to have analytical procedures implemented in all laboratories, and backup samples to repeat the quantification, instead of centralising the analytics within a single laboratory. Even if this adds a second source of variability, it avoids problems due to sample transference and/or problems with the analytical equipment in one laboratory.

Next Steps

Analysis of the permeability concentration dependence of the compounds in order to characterise the passive diffusional permeability and the michaelis menten parameters Km and Vmax, and comparison of these results among laboratories as well as the correlation with the markers controls.

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EXERA

Development of 3D *in vitro* models of estrogen-reporter mouse tissues for the pharmaco-toxicological analysis of nuclear receptors-interacting compounds (NR-ICs)

Contract number:	LSHB-CT-2006-037168
Project type:	Specific Targeted Research Project (FP6)
EC contribution:	€ 2 173 492
Starting date:	1 October 2006
Duration:	42 months

Website: http://www.altaweb.it/exera/index.html

Background

Industries from different fields (pharmaceuticals, chemicals, cosmetics, foods and toxicologicals) need reliable, fast, and economical *in vitro* models as alternatives to animal testing. These models should be able to provide predictive data on the effects of Nuclear Receptor-Interacting Compounds (NR-ICs), and in particular, Estrogen Receptor-Interacting Compounds (ER-ICs) on animal and human health.

Risk assessment needs are increasing for the following reasons:

- First, there is an increasing number of drugs with endocrine action (mainly estrogens/antiestrogens for women; 80% of the marketed drugs are directed to women, such as birth control agents, cancer therapy and prevention, and hormone replacement therapies (Hartung 2001)).
- Second, the number of xenobiotic compounds produced during industrial processes is increasing. They are contaminating the environment, and have structures that indicate that they might interact with male and female endocrine systems (Reporter..,2004; http://europa..; Charles, 2004).
- Third, research evidence is proving the limitations of several non-mammalian models to predict the physiological consequences (risks and benefits) linked to the exposure of humans to ER-ICs (Barton, 1998). Therefore, the development of ap-

propriate *in vitro* models that can reproduce features and reactivity of specific mammalian target tissue/organs to ER-ICs has become an urgent research priority. The scientific, economic, social and ethical issues are therefore considerable.

State-of-the-Art (and Major Drawbacks of Existing In Vitro Models)

The tissue- and organ-specific *in vitro* models that have been developed to date have several serious limitations:

- 2-dimensional culture conditions may not be optimal for modelling tissue-like organisation, cellular functions, and differentiation of mammalian organs. For example, the polarised cells of a parenchymal tissue, which normally require complex cellular interactions, cannot behave physiologically when adhering to solid substrates, as in the case of conventional culture conditions. Cell-cell and extracellular matrix-cell interactions play a fundamental role in maintaining the function of numerous organ systems. Hence, tissue engineering may provide opportunities to overcome the limitations of monolayer cultures, and to maintain tissue-like architecture and functionality.
- The systems used for *in vitro* and *in vivo* analysis of NR-ICs (mainly estrogens and androgens) are generally composed of cells derived from reproductive tissues. The recent knowledge of the widespread distribution of nuclear receptors (in particular steroid receptors), in all tissues of the organism, and their involvement in several diseases, makes the available systems inadequate to assess the effects of NR-ICs on whole organism physiology.

Objectives

To overcome the above-mentioned deficiencies, six industry representatives have agreed to collaborate with three public research organisations to achieve the following objective: The development of novel *in vitro* 3D models of mouse tissues for the pharmaco-toxicological analysis of ER-ICs for liver, skin, bone (non-reproductive systems), ovaries and testis (sex-specific reproductive systems).

This approach involves the following systems/approaches:

- *Immortalised cells from tissues of reporter and/or wild-type mice:* Numerous studies performed on cells from this animal model in several laboratories have demonstrated its reliability and suitability to the study of molecules acting through estrogen receptors. This mouse model represents a new strategy that allows the study of estrogen receptor-mediated gene regulation *in vivo* and in derived *in vitro* systems.
- *3D cultures:* A microgravity RCCS (Rotating Cell Chamber System) device has been developed and used for the culture of cells in 3D. We have shown

that it maintains cells in more physiological conditions, and in a more differentiated state compared to conventional 2D cultures. In EXERA, we have applied the RCCS system to the maintenance of cells with a sensitive estrogen-responsive phenotype.

- *In vivo*: The reporter mouse model has also been exploited for the pharmaco-toxicological analysis of selected ER-ICs *in vivo,* with protocols that allow the fulfillment of two of the 3Rs (reduction and refinement).

Experimental Design

Tissues from transgenic reporter mice and wild-type mice have been placed in culture and the cells immortalised. After clone selection and expansion, the cells obtained from liver, skin, bone, testis and ovaries have been expanded and established as cell lines. These have been tested in culture for the expression of a differentiated phenotype and their response to ER-IC. Tests have been performed in 2D and 3D cultures. Results for the expression of differentiation pathways and estrogenic responses have been compared to identify the best culturing method. The reporter mouse model has been applied to the pharmaco-toxicological analysis of selected ER-ICs *in vivo,* and protocols for *in vivo* imaging have been established. Chronic exposures and longitudinal studies have been performed and standardised.

Results

Status

Most of the deliverables have been accomplished in due time. For some of them, a 6-month extension was requested (total duration of the project: 42 months).

1. The cell immortalisation processes from five mouse tissues permitted the successful generation of a number of immortalised cell clones. Some of them required more time to produce, presumably because of specific characteristics associated with different tissues. The consortium generated three clones from skin fibroblasts, three clones from liver cells, two clones from bone marrow mesenchymal stem cells, three clones from Sertoli cells, and four from granulosa cells. The generation of these cell clones not only provided further *in vitro* cell systems for the pharmaco-toxicological analysis of ER-ICs, and eventually of other chemicals, but also allowed a comparison of the properties of 2D and 3D culture methods of the different tissues in order to identify advantages/drawbacks of the 3D over the conventional 2D methods. These *in vitro* systems were also compared to *in vivo* tissues in the mice. Not all the cell clones were responsive to estrogens; however, as reported below, the 3D RCCS bioreactor increased the sensitivity of cells to estrogen.
2. EXERA was mainly focused on the investigation of a new 3D culture sys-

tem based on microgravity (RCCS) for its possible application to the pharmactoxicological analysis of chemicals (with a specific focus on endocrine disruptors). EXERA has demonstrated that the original idea was correct—namely, that 3D cell cultures (involving mainly the RCCS) produce cells expressing a more differentiated phenotype (Figure 1).

3. Standardisation and optimisation of the 3D assays are in a preliminary stage. However, EXERA demonstrated that cells cultured under microgravity conditions show a phenotype that more closely resembles the *in vivo* situation.
4. The RCCS bioreactor has also been characterised with respect to the response of cells to estrogenic chemicals (a focus of the EXERA programme). Cells cultured in microgravity conditions express a more reactive estrogen responsive phenotype (Figure 3). We have

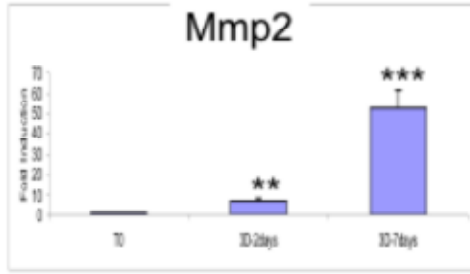

Figure 1. Skin fibroblasts grown in 2D and 3D cultures. Mmp2 expression as a marker of differentiation.

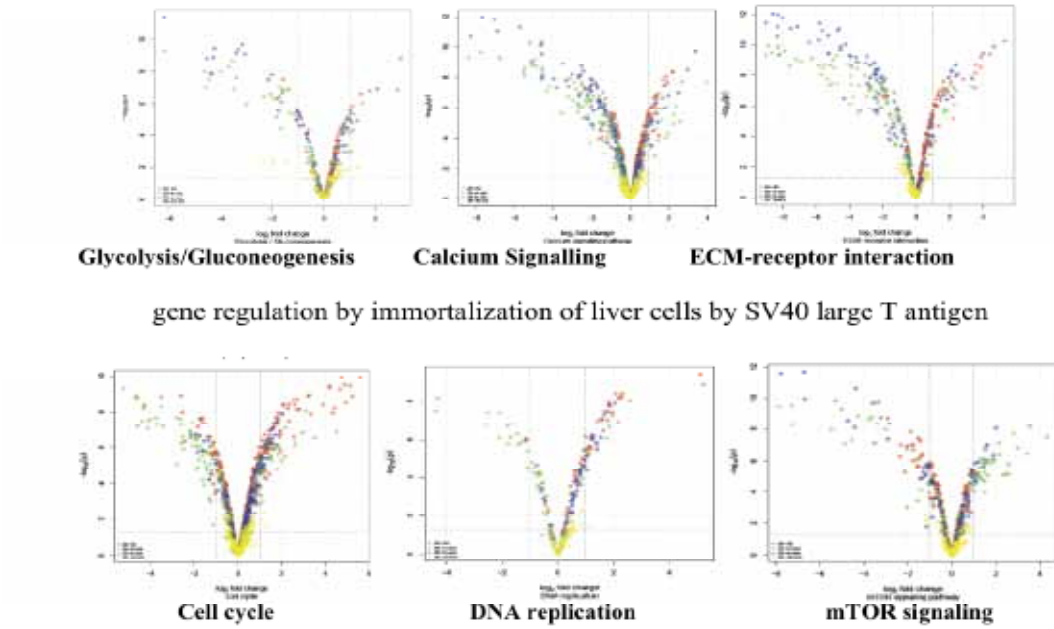

Figure 2. Whole genome gene expression profiles (microarrays). Metabolic pathways in liver cells. Results show changes in gene expression in immortalised cells and the changes occurring when switching the cells from 2D to 3D (RCCS) culture conditions.

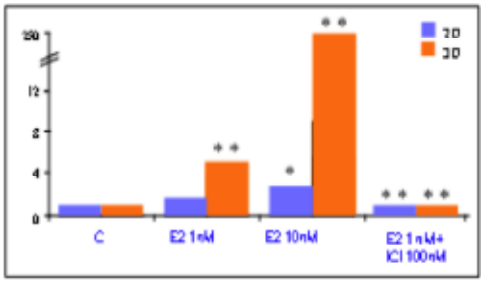

Figure 3. A comparison of estrogen inducibility of the reporter in skin fibroblasts grown in 3D (orange) versus 2D (blue) cultures.

indicated that this is due to an up-regulation of estrogen receptors (ERα and ERβ) in cells in 3D. Cell responses following 2D and 3D culturing and tissue responses *in vivo*, were characterised by the use of different estrogenic chemicals. The time course of this response have been detailed and optimised.

5. The generated cell systems will not be forwarded for validation for conventional 2D assays, but these assays served as necessary tools for establishing appropriate conditions for 3D testing protocols. However, some of the generated clones from mesenchymal stem cells (that differentiate into: osteoblasts, adipocytes and chondrocytes) have some unique characteristics, even in a 2D environment (very few such systems are available). Financing the validation steps of one of the unique bone-derived cell lines (WT6 or ERE22) is under evaluation.
6. The optimised RCCS 3D method has been compared to 2D cultures through extensive 2D-3D comparisons using micro- and macr-oarrays, tissue arrays, and reporter assays using 10 chemicals with endocrine disrupter activity. We are confident that the system can be developed for application for routine analysis. The technical and economic commitment of the producing company (CELLON) to develop the RCCS device into a routine instrument for pharmaco-toxicological analysis will be an important issue. Improvement of the RCCS has been initiated in one of the partner's laboratories in collaboration with the industrial partners as a self-supported continuation of the work performed in EXERA.
7. Chemicals from different classes of endocrine disruptors (EDs) and drugs have been tested in EXERA as follows:
   - *Physiological estrogens:* estradiol (control) (1)
   - *Drugs:* Raloxifene (2) and two to be determined. Compounds x (3) and y (4) from Partner 6.
   - *Environmental pollutants:* Bisphenol A (plasticiser) (5), ßBHC (pesticide) (6), cadmium (heavy metal) (7)
   - *Food components:* genistein (8), daidzein (9) and resveratrol (10).
8. We also tested the following Selective Estrogen Receptors Modulators (SERMs): bazedoxifene (Baz), clomiphene (Clo), enclomifene (Enc), fispemifene (Fis), compound HM-3000, compound HM-320A, lasofoxifene (Las), ospemifene (Osp), raloxifene (Ral) and tamoxifen (Tam). The assays employed were 2D and 3D cell culture, and the ERE-LUC reporter mouse model (*in vivo*)

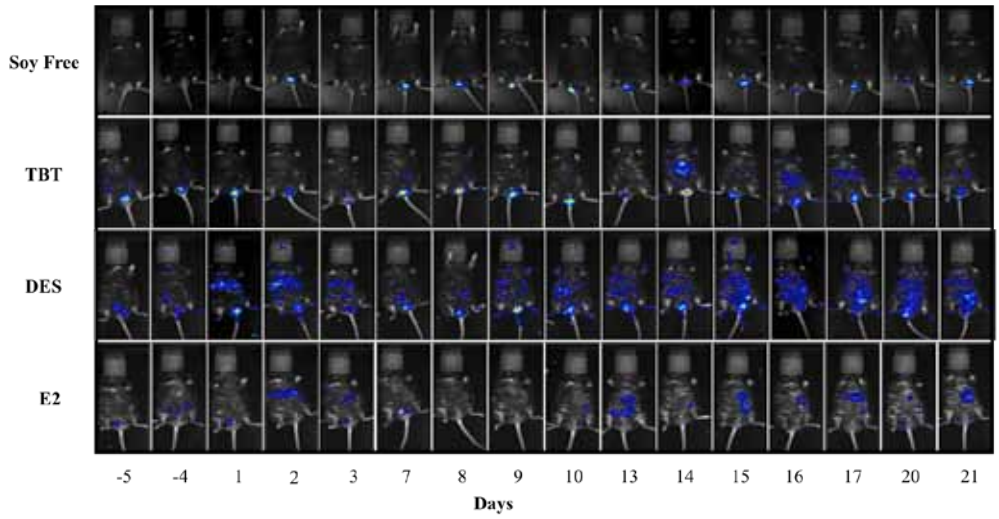

Figure 4. Longitudinal studies on luciferase expression in ERE-LUC mice exposed to estrogens. Imaging has been performed by the NightOwl system (Berthold).

depending on the tested chemical. Extensive pharmaco-toxicological analysis and physiologically-based pharmacokinetics have been performed in these models.

9. Specific assays performed and chemicals tested to delineate estrogen-specific signatures include the following:
   - *Skin fibroblasts (clone PE6).* 2D and 3D comparisons on all the above-indicated chemicals and drugs. (SERMs synthesised by the partner HORMOS.)
   - *Liver cells (clone MIIF).* 2D and 3D comparison on E2, gensitein, BPA *in vitro* and drugs. (SERMs synthesised by the partner HORMOS.)
   - All the above-indicated chemicals and drugs (SERMs) were also tested *in vivo* in the ERE-LUC reporter mouse model (the protocol is available).
   - *Bone osteoblasts (clone WT6 and ERE22) and the osteosarcoma U2OS:* E2 and drugs. (SERMs synthesised by the partner HORMOS.) Bone responses were also measured in the ERE-LUC reporter mouse model.
   - *Sertoli cells (clone Q8R).* 2D and 3D assays for E2, genistein, and BPA, plus other responses to ER-ICs.
   - *Granulosa cells (clone GC1):* 2D assays for E2, EE2, genistein, and DDE, plus other responses to ER-ICs.

Beyond the data generated in *in vitro* systems, the EXERA consortium also exploited the use of the mouse estrogen reporter transgenic model (ERE-LUC). Two of the 3Rs principles (refinement

and reduction) could be fulfilled using mice for pharmaco-toxicological testing. Estrogenic chemicals have been tested in acute, and for the first time, in longitudinal studies (Figure 4), by coupling the mouse model to *in vivo* imaging technologies. The results generated illustrate the pharmacokinetics and pharmacodynamics of these compounds.

Differential analysis of the generated cell clones under different conditions, as well as the use of the transgenic mouse model ERE-LUC, expressing a hormone regulated luminescent reporter, produces the results observed in Figure 2.

Problems

Not all the tissues when placed *in vitro* in 2D cultures respond optimally to estrogens. The major identified reason is a down-regulation of estrogen receptor expression. One solution is to transfect the established cell lines with ER genes to confer inducibility by ER-ICs. Alternatively, when placed in 3D cultures in the RCCS, the cell lines do respond more sensitively to ER-ICs, and an up-regulation of estrogen receptors is observed (up to 3-fold). The response to ER-ICs is increased.

Next Steps

The project concluded on 31 March 2010. The results to date support the claim that cell lines cultured in 3D in the microgravity bioreactor RCCS express a more differentiated phenotype, and some of them are more sensitive to ER-ICs. This 3D culture method represents an innovative system for the pharmacotoxicological testing of environmental estrogens, hormonal drugs, and food components.

The results of the EXERA project will allow the adaptation of the RCCS for the pharmaco-toxicological analysis of chemicals.

Partners 1 and 3 are searching for industrial partners to commercialise 2D assays based on the generated cell lines. These cell lines can be used in pharmaco-toxicological screenings of drug candidates, as well as for toxicological purposes.

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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
Starting date:	1 January 2007 (finished)
Duration:	36 months

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

Objectives

The objective of INVITROHEART was to establish stable cell lines that reliably reflect human cardiomyocyte properties through the development of an *in vitro* model that is derived from human embryonic stem cells (hESC). The aim was to deliver a trustworthy *in vitro* model that would be accepted by regulatory authorities and 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 EU regulation on the Registration, Evaluation and Authorisation of Chemicals (REACH). 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 chemical toxicity testing, replacement of animal test methods can also be realised in the cosmetics, food, and commodity chemicals industries.

The means to accomplish the project objectives were, in addition to new stable hESC-derived cardiomyocytes (hESC-CM), (1) state-of-the-art methods for electrophysiological cardiac cell monitoring, (2) optical micro-sensor monitoring in micro-cultivation systems for *in vitro* screening, and (3) a multi-micro-bioreactor platform for high-throughput screening of drugs and chemicals. Comparative studies of hESC-CM with established *in vitro* models were carried out in order to validate the new models and methods.

The clinical expertise in the project was represented by a leading European university hospital and a well-known European pharmaceutical company. The four participating European SME companies, very experienced in life science and microsensor technologies, were the key providers of state-of-the-art technology. Another important partner for the project is the European Centre for Validation of Alternative Methods (ECVAM).

Experimental Design

The experimental work in INVITROHEART was focused on improving the procedures for differentiation of hESC towards functional cardiomyocyte phenotypes, and their subsequent expansion and application for testing. Much effort was concerned identification of biomarkers for cardiomyocyte differentiation, and a thorough characterisation of cells after differentiation through improved culturing protocols (Table 1).

Advanced *in vitro* methods for assessment of the cardiotoxic potential of drugs and chemicals were evaluated and adapted for use with hESC-derived cardiomyocytes (hESC-CM). Several endpoints were evaluated with focus on electrophysiology parameters (e.g., QT-prolongation), representing functionality as the most critical toxicology parameter. Additional parameters identifying cardiotoxic effects on the cellular level, such as oxygen consumption measurements, metabolic activity, membrane integrity, mitochondrial transmembrane potential, and intracellular calcium flux, were supplementing the testing platform to enhance the predictability.

The test methods were benchmarked with reference cardiomyocytes to compare the capacity of hESC-CM with alternatives. Furthermore, new biosensor technologies were evaluated for use in cardiotoxicity screening, e.g., analysis of troponin release with surface plasmon resonance technology.

Results

The most significant achievements 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 have been characterised and the cells express high levels of ion channels and other cardiac markers demonstrating successful differentiation into a cardiomyocyte phenotype (Table 1).
- 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 estab-

Table 1. Examples of genes up-regulated in cardiomyocyte clusters including cardiomyocyte specific genes, as well as transcription factors which indicate cardiomyogenic differentiation, and genes for ion channels commonly used in cardiotoxity testing.

Gene Symbol	Gene Title
Cardiac Associated	
MYH6	myosin, heavy polypeptide 6, cardiac muscle, alpha (cardiomyopathy, hypertrophic 1)
MYL7	myosin, light polypeptide 7, regulatory
NPPA	natriuretic peptide precursor A
TNNT2	troponin T type 2 (cardiac)
ANXA1	annexin A1
MYBPC3	myosin binding protein C, cardiac/myosin binding protein C, cardiac
Transcription factors	
NKX2-5	NK2 transcription factor related, locus 5 (Drosophila)
GATA4	GATA binding protein 4
MEF2C	MADS box transcription enhancer factor 2, polypeptide C (myocyte enhancer factor 2C)
HAND1	heart and neural crest derivatives expressed 1
ISL1	ISL1 transcription factor, LIM/homeodomain, (islet-1)
Ion channels	
KCNH2	K voltage-gated channel, subfamily H (eag-related), member 2, (long QT syndrome 2)
KCNQ1	K voltage-gated channel, KQT-like subfamily, member 1
KCNA5	K voltage-gated channel, shaker-related subfamily, member 5
KCNJ3	K inwardly-rectifying channel, subfamily J, member 3
KCNJ2	K inwardly-rectifying channel, subfamily J, member 2
CACNA1C	Ca, voltage-dependent, L type, alpha 1C subunit

lished 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
- 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.

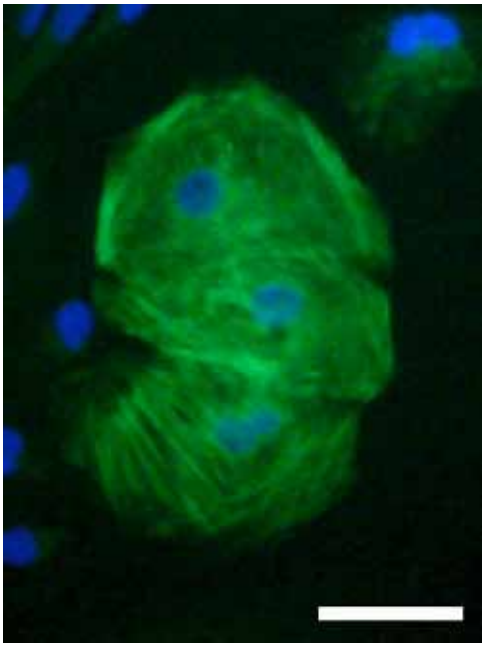


Figure 1. Dissociated hESC-CM stained for the cardiac specific protein troponin, indicating that phenotype is retained when cardiomyocytes are in a single cell format (troponin, green; nuclei, blue; scale bar, 50 µm). This shows potential for use in single cells applications, such as voltage clamp analysis and immunohistochemistry.

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 (Figures 2 and 3). 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

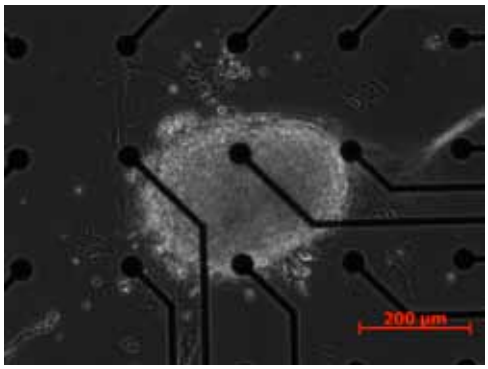


Figure 2. hESC-CM cluster on a multi-electrode array (MEA) plate for field potential recordings. The cells are from partner Cellartis AB and the MEA system is from partner Multi Channel Systems GmbH.

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 of reference compounds, the cells are characterised and ranked for suitability in safety screening. Figure 4 shows a drug-induced prolongation of the cardiac field potential by the hERG channel antagonist E-4031. This channel is a very important target in safety pharmacology, and its presence is crucial.

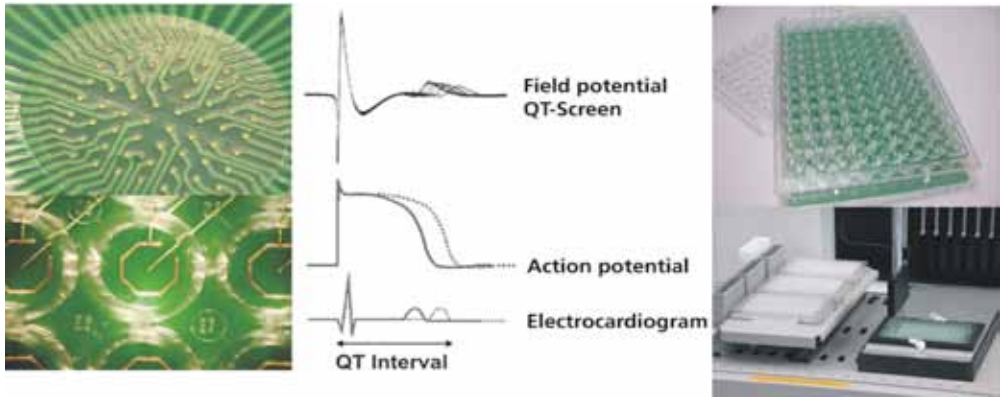


Figure 3. In the left panel the transition from the 60 electrode ecoMEA to a single electrode in a well of a 96-well plate is shown. The middle panel aligns an actual QT-Screen recorded field potential with illustrations of corresponding action potential and electrocardiogram measurements under control conditions and with prolonged QT interval.

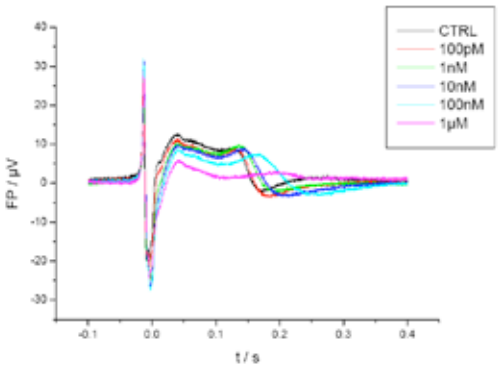
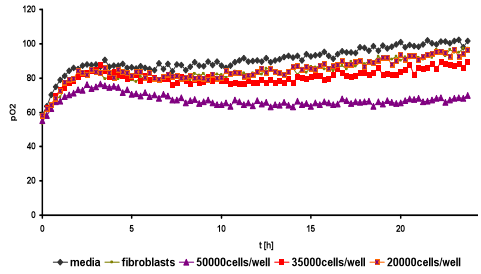


Figure 4. Field potential recordings from hESC-CM with MEA technology showing dose-dependent increase in re-polarisation time (QT-prolongation) by the hERG-blocker E-4031.

An aluminum holder for small plastic vessels (from 96-well strip plates) with integrated optical oxygen sensors was tested and evaluated for use with the SensorDish Reader for respiration measurements (Figure 5). 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.

This set-up was tested on hESC-derived cardiomyocytes seeded as monolayers, and some results are presented in Figure 6.

Figure 5. 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.



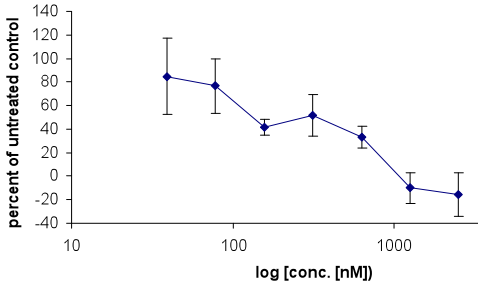


Figure 6. a) hESC cardiomyocytes as monolayers (beating) in small plastic vessels shown in Figure 5; b) Dissolved oxygen measurement using different number of seeded hESC cardiomyocytes per well over a period of 25 hours; c) dose-response curve for doxorubicine tested on dissociated hESC cardiomycytes in the set-up shown in Figure 5.

The uptake of substrates and secretion of metabolites (amino acids, lactate, glucose and pyruvate) were measured by HPLC. The flux map was further optimised. In an experiment, the HL-1 cells maintained in Claycomb medium with FCS and those maintained in Claycomb containing CCT were compared (Figure 7).

The uptake of substrates and secretion of metabolites (amino acids, lactate, glucose and pyruvate) was measured by HPLC. The uptake/production rates of metabolites were compared with the control. These are used for the estimation of intracellular fluxes with the help of a simplified metabolic network model (Figure 8). In the experiment with Verapamil, the first conclusion that can be drawn is that despite a reduced uptake of glucose, the TCA-cycle remains unaltered by verapamil. Therefore, a lower amount of glucose-derived pyruvate can be metabolised to lactate whilst the glucose uptake is reduced. The exometabolome data were analysed by principal component analysis (PCA) (Figure 9). It is observed that the test usually clusters away from the control, and also showed a trend for separation, such as in case of doxorubicine (Figure 9a).

In addition, labelling studies for detailed flux studies were also carried out. Glucose/lactate/amino acid uptake and production of whole hESC-CM clusters was determined. Different numbers of clusters were seeded in 384-well plates in a total volume of 80 µl. Glucose in the medium was replaced with [U-$^{13}C_6$] glucose. After 96-hours incubation, the supernatant was collected and analysed for glucose, lactate, and all 20 amino acids. In addition, ^{13}C-enrichment of produced lactate was determined (Figure 10).

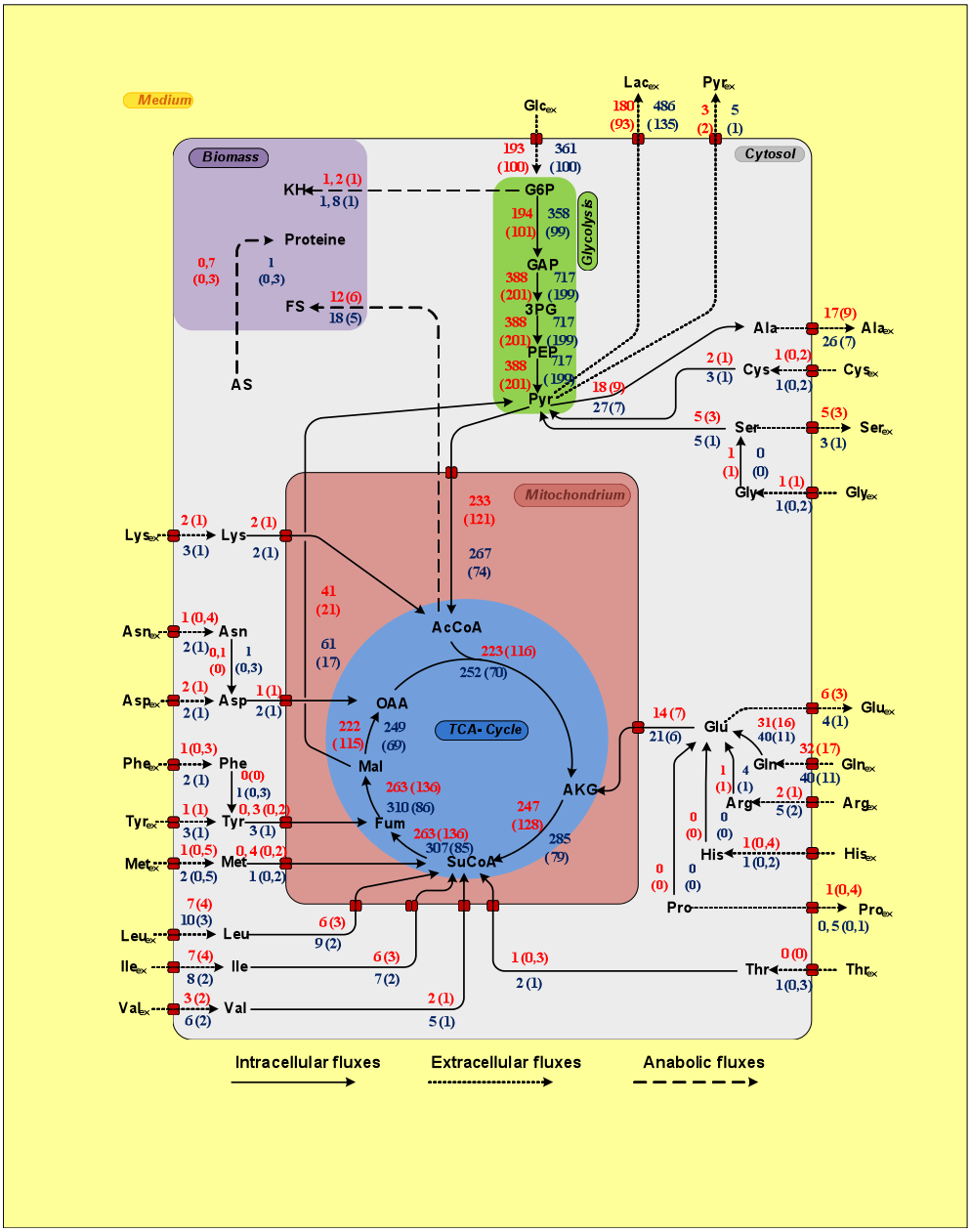


Figure 7. Flux map of HL-1 cells. The numbers indicate the specific rates in fmol/cell*h, while the numbers in bracket are values normalised to glucose uptake. Numbers in red indicate growth in CCT medium, whereas the numbers in blue indicate growth in Claycomb medium containing FCS.

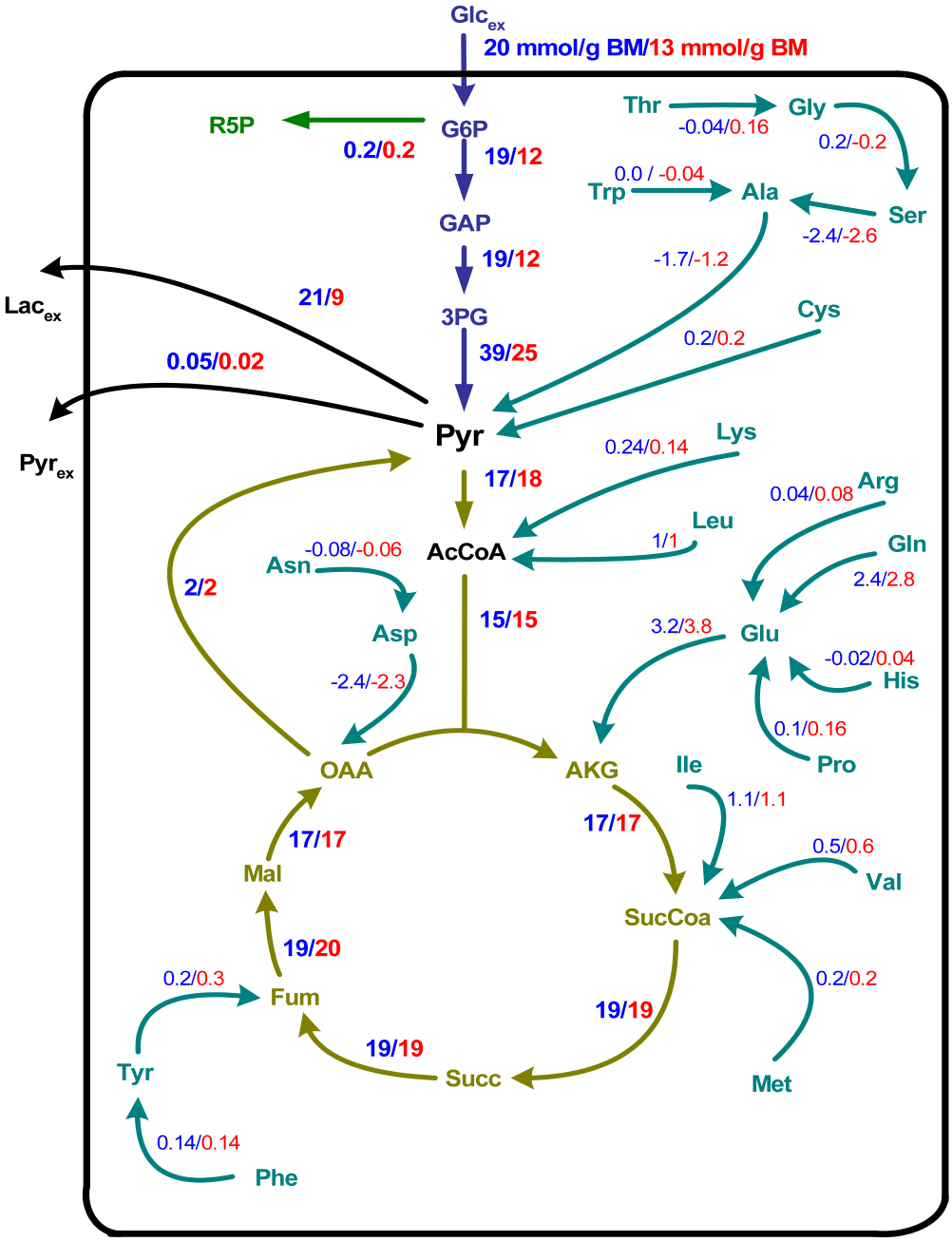


Figure 8. Simplified metabolic network model for HL-1 cells. The blue numbers show the fluxes of untreated cells, while the red numbers indicates fluxes of verapamil (4μM) treated cells. The unit of all fluxes is mmol/g biomass.

As is apparent from Figure 10, the standard deviation in glucose and lactate rates decreases with increasing cluster numbers. This is probably due to varying cluster sizes. The lactate/glucose ratio therefore shows huge variations for lower cluster numbers. For six clusters per well, the ratio ranges between one and two. Thus, a high amount of glucose carbon seems to be secreted as lactate. This is also observed from the ^{13}C-labelling data of lactate (Figure 11). No matter how many clusters were seeded, the production rate of fully labelled lactate (m+3) dominates the overall molar lactate mass isotopomer rates and accounts for approximately 80-90%. Thus, most of the produced lactate carbon is directly derived from glycolysis and did not enter the TCA-cylce. Unlabelled lactate, which accounts for 10-20%, is already present in hESC-CM medium due to addition of fetal bovine serum. Lactate, m+1 and m+2, account for 1-5%, and seem to be derived either from pentose phosphate pathway or TCA-cycle. Increasing anaerobic glycolysis may result due to hypoxic conditions, e.g., limitation of oxygen after long period of testing. Another reason for increased anaerobic glycolysis may be the presence of unknown carbon sources in FCS, such as free fatty acids. High amounts of fatty acid-derived acetyl-CoA can inhibit pyruvate decarboxylation.

In brief, the labelling studies show that there are significant differences between the central metabolism of hESC derived cadiomyocytes and the HL-1 cells. Further analysis of data is in progress.

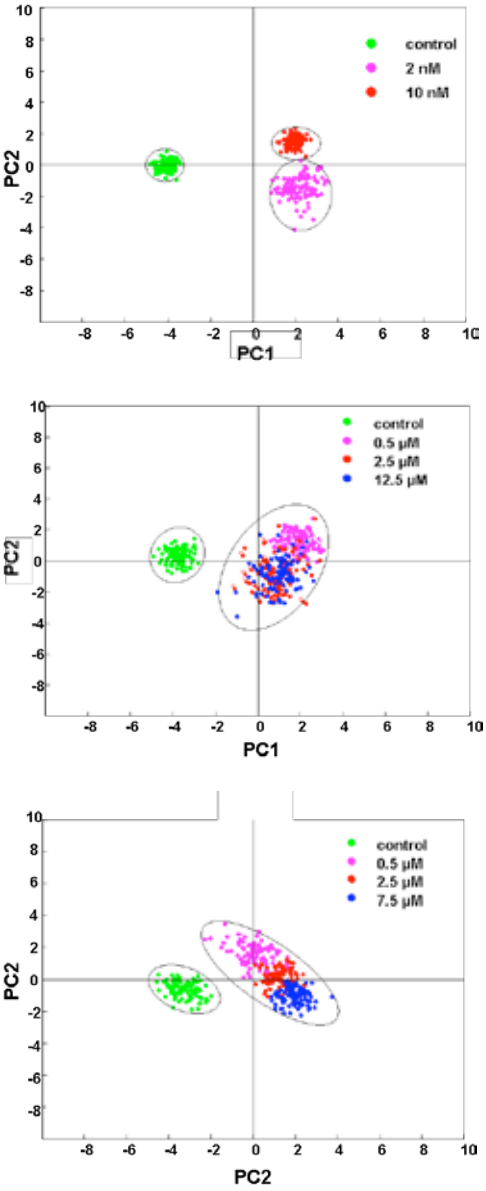


Figure 9. PCA for the test compounds (top to bottom: Doxorubicine, Verapamil, Haloperidol). Green dots indicate the control, whereas the pink, red and blue indicate increasing concentrations of respective test compounds.

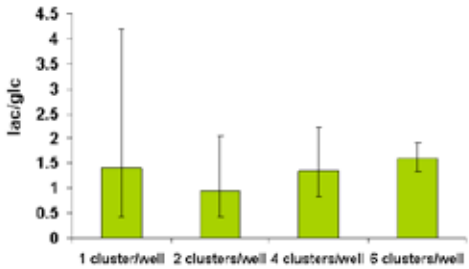


Figure 10. Ratio of lactate production rate and glucose uptake rate of hESC-CM clusters.

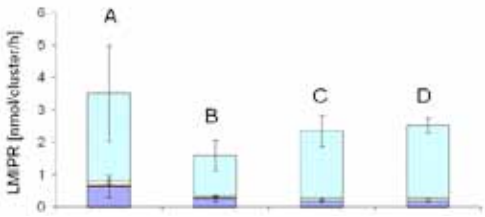


Figure 11. Lactate mass isotopomer production rate in: A) 1, B) 2, C) 4, and D) 6 hESC-CM clusters.

Next Steps

Now that the INVITROHEART project is finished, the main remaining action is to make the results available for further studies. In addition to the published scientific reports and reports under submission, an invited review article will be submitted that will comprehensive summarises the results. The partners will support **AXLR8** and other actions taken to disseminate the INVITROHEART results.

Publications

1. Meyer T, Sartipy P, Blind F, et al. (2007). New Cell Models & Assays in Cardiac Safety Profiling. *Expert Opin Drug Metab Toxicol.* 3, 507-17.

2. Sartipy P, Björquist P, Strehl R, et al. (2007). The application of human embryonic stem cell technologies to drug discovery. *Drug Discovery Today* 12, 688-99.

3. Synnergren J, Adak S, Englund MC, et al. (2008). Cardiomyogenic gene expression profiling of differentiating human embryonic stem cells. *J Biotechnol.* 134, 162-70.

4. Björquist P, Sartipy P, Strehl R, et al. (2008). Human ES cell derived functional cells as tools in drug discovery. *Drug Discovery World (Winter 2007/08)* 9, 17-24.

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6. Stummann T, Beilmann M, Duker G, et al. (2009). Report and recommendations of the workshop of the European Centre for the validation of alternative methods

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9. Andersson H, Kågedal B, Mandenius CF (2010). Surface plasmon resonance measurement of cardiac troponin T levels in cardiomyocyte cell culture medium as indicator of drug-induced cardiotoxicity. *Anal. Biochem. Chem. 398,* 1395-1402.

10. Andersson H, Steel D, Asp J, et al. (2010). Assaying Cardiac Biomarkers for Toxicity Testing using Biosensing and Cardiomyocytes Derived from Human Embryonic Stem Cells. *J. Biotechnol. 150*, 175-81.

11. Bergström G, Mandenius CF (2010). Immunosensor assay for troponin and albumin using orienting antibody affinity ligands and surface plasmon resonance. [Submitted for publication].

12. Kajic K, Brantsing C, Jonsson M, et al. (2010). ISL1+ subclones derived from rhabdomyosarcoma cell lines as an *in vitro* model for human cardiac progenitor cells. [Submitted for publication].

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 and intestine *in vitro* models for pharmacokinetics and pharmacodynamics studies

Contract number:	LSHB-CT-2006-037499
Project type:	Specific Targeted Research Project (FP6)
EC contribution:	€ 2 933 291
Starting date:	1 January 2007
Duration:	36 months

Website: http://www.liintop.cnr.it

Background

The EU regulation for the Registration, Evaluation and Authorisation of Chemicals (REACH) implies, on one side, a cumbersome plan of testing, and on another side, the use of a huge number of animals. The European Centre for Validation of Alternative Methods (ECVAM) has already addressed the possibility of using alternative methods, according to the 3Rs principle, in order to reduce this number, or at least the animal suffering associated with certain kinds of tests. The EU has addressed this issue in the Directive 86/609. The use of non-validated alternatives has also been suggested based on a "weight of evidence" approach, i.e., widely used and well consolidated procedures.

Moreover, the scientific relevance of the *in vitro* studies in toxicology has been acknowledged by several scientific institutions[1-2] for the possibility they offer to work on human systems and at cellular and molecular levels. This will allow understanding specific mechanisms-of-action by different compounds.

In this context, a successful outcome of the project will have a strong and diversified impact on social and economic issues. In fact, optimised *in vitro* systems comply

[1] Anon. (2000). Toxicology for the Next Millennium. Proceedings of a conference. September 20-23, 1999, Warrenton, Virginia, USA. *Ann N Y Acad Sci.* 919, 1-324.

[2] US National Research Council (2007). *Toxicity Testing in the Twenty-First Century: A Vision and a Strategy*. Washington, DC: National Academies Press.

with the request of reducing animal experiments, thus satisfying a widely shared ethical concern. On the other hand, they offer economic advantages in terms of reduced time consumption and lower costs in safety assessment of novel drugs.

Objectives

The main aim of the project is to provide optimised 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 into five main areas as follows:

1. Development of new *in vitro* models; determine which of the existing advanced *in vitro* liver and intestinal models are most appropriate and provide improved performance in the screening and testing of new drugs' absorption and metabolism:

    - Comparison of selected functions with the corresponding normal human tissue *ex vivo* (i.e., primary human hepatocytes or human intestinal epithelium).
    - Optimisation of the culture conditions to make the models stable over time for specialised functions; therefore, a new strategy using chromatin-remodelling agents (histone-deacetylase inhibitors) will be used for primary hepatocyte-based models.
    - New approaches to generate metabolically-competent human hepatic cell lines (HepG2, HepaRG); this will include genetic manipulation of existing cell lines (HepG2), which will be transfected with key transcription factors in order to allow an appropriate expression of the differentiated phenotype.
    - Development of more complex cell co-culture models to combine absorption and metabolism in the intestines and liver.
    - Optimisation of the culture conditions to differentiate adult bone marrow stem cells into functional hepatocytes.

2. Identification of liver and intestinal *in vitro* models better expressing drug transport and metabolism:

    - Modulation by culture conditions for their expression.
    - 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 cellular basic functions and structures (e.g., mitochondrial membrane potential, lipid peroxidation, intracellular calcium concentration, DNA content, determination of nuclear area, and cell number and viability.
    - Effects on differentiated functions: steatosis, cholestasis, phospholipi-

dosis, absorption, etc.

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

    - Development of mechanism-based pharmacokinetic models.
    - Exploration of predictive utility of new *in vitro* models.
    - Identification of areas requiring refinement for future *in vitro* models.

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

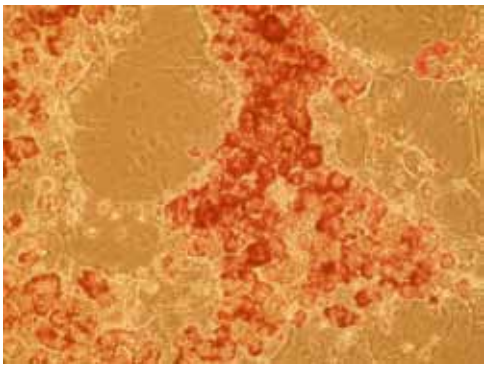


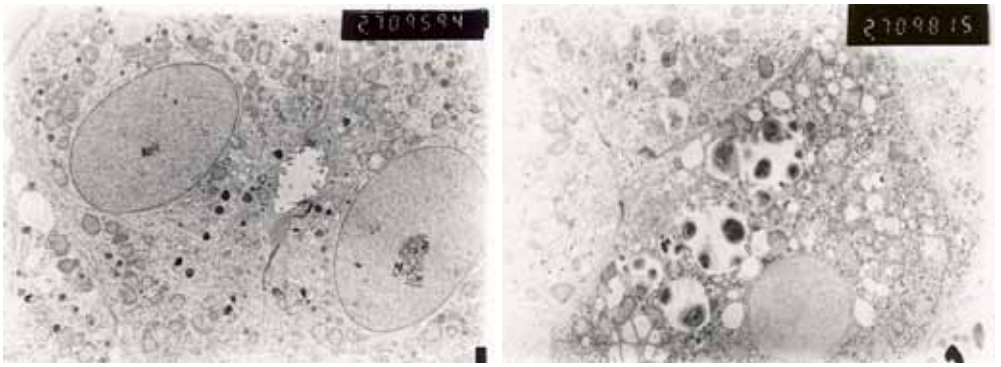
Figure 1. Accumulation of lipid droplets (steatosis) in HepaRG cells after a 14-day treatment by 50µM tetracycline. Oil-red O staining.

Experimental Design

To achieve the proposed goals the following methodologies will be employed:

- hepatocyte and enterocyte *in vitro* cultures.
- genomic and proteomic methods.
- biotransformation and metabolic as-

Figure 2. Accumulation of lamellar bodies (LB), hallmark of phospholipidosis and lipid droplets (LD) (steatosis) in HepaRG cells after a 14-day treatment by 20µM amiodarone (right; image at left is control). Electron microscopic micrograph.



says in the two cell types.
- transport measurements in intestinal cell lines (passive and active).
- analytical techniques by LC-MS and MS-MS.
- mechanism-based pharmacokinetic modelling.
- statistical data handling.

Results

Status

The official end date of the project was 30 June 2010.

The main goals concerning cell cultures have been achieved:

- Human hepatocytes *in vitro* (HepaRG) are available, very close to the *in vivo* situation, expressing most of the functional and genotypic characteristics of normal hepatocyte. Cell culture and maintenance protocols have been optimised. Higher-throughput assays with minimised resource needs has been developed both for metabolism and permeability evaluation of new chemical entities and their predictivity for *in vivo* human situation assessed similar or better than traditional methods.
- Cryopreserved HepaRG cells, differentiated to express high levels of drug-metabolism enzymes and transporters, have been produced, and their performance to quantitatively predict human intrinsic clearance proved as good as the actual industrial standard, i.e., pooled cryopreserved human hepatocytes.
- Other approaches to develop functional models of *in vitro* human hepatocytes have progressed; induction of stem cell differentiation by TSA.
- The Caco-2 cells have been shown to be very close to the human normal small intestinal cells by genomic and functional analysis. Optimised culture conditions have been defined that allow strict control over the process of differentiation to obtain the best expression of differentiation markers.
- In addition, replacement of fetal bovine

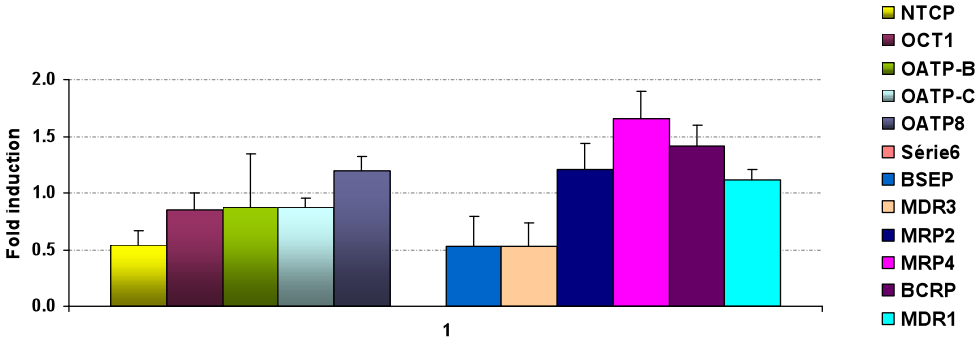


Figure 3. Effects of chlorpromazine on uptake and efflux transporters in HepaRG cells. Specific inhibition of NTCP, BSEP and MDR3 and overexpression of MRP4 and BCRP as observed in cholestasis (mRNA levels, 24h treatment with 50µM chlorpromazine).

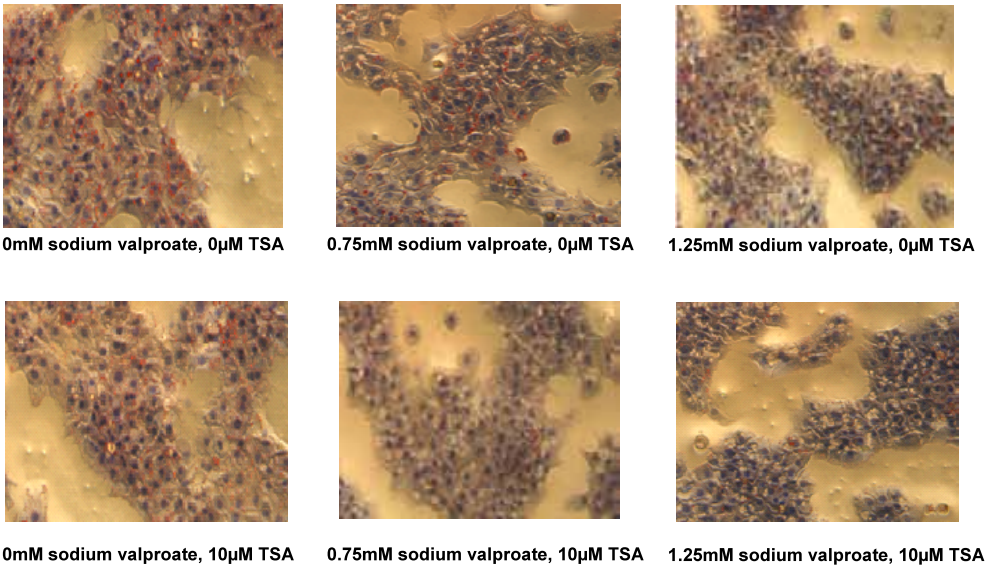


Figure 4. Analysis of intracellular neutral lipid accumulation by Sudan (red lipid droplets) staining. Primary hepatocytes were exposed for 72-h to sodium valproate (0-1.25mM), in the absence and presence of 10µM TSA. Original magnification 20x 10. n=3.

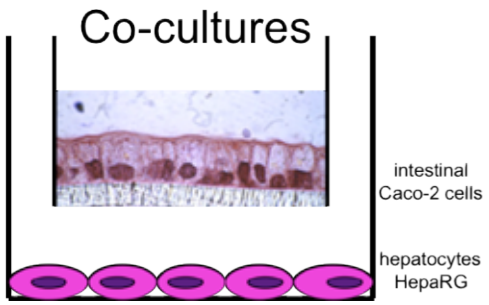
serum from the culture medium with a chemically-defined supplement allows optimal differentiation of the cell line under better controllable condition.
- Co-culturing of intestinal and liver cells is giving promising results and is undergoing further optimisation.

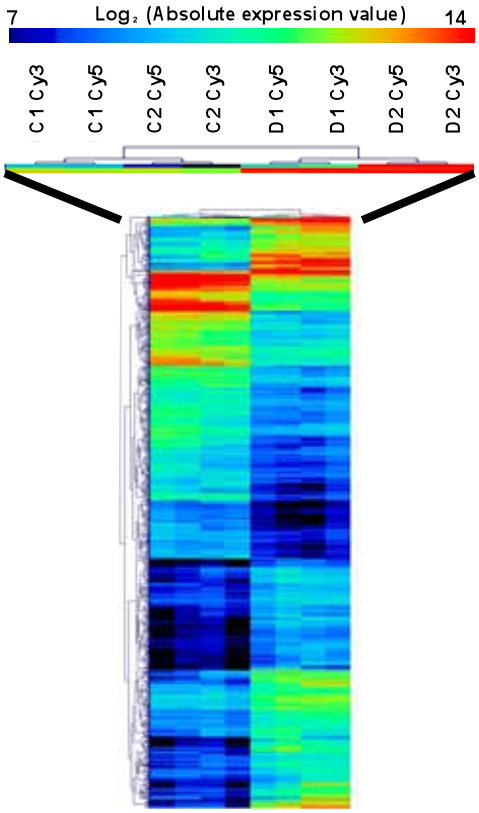
The main goals in the area of absorptive and metabolic functions have been achieved:

- Both cell types have been extensively characterised for transporters and Phase I and Phase II enzymes.

In the area of molecular and cellular targets, an effective strategy has been set up:

- By using the new technology of HCA, it has been implemented a reproducible high-content multiparametric cytotoxicity assay, based on the measurement of multiple parameters that are morphological and biochemical indicative of prelethal cytotoxic effects, representative of different mechanisms of toxicity, at the level of single cells and that





allows a high-throughput screening. For oxidative stress—an eluding issue that often emerges in late phases of drug development process, with major losses for the pharmaceutical industry—a miniaturised assay was developed assessing 8-ISP, GSH, 8-oxo-dG, and α-GST with significant reduction in assay time, increase in throughput, and with the latter marker, coverage of both centrilobular and periportal regions of the liver.

- Assays of a restricted number of compounds on both cell types, will assess the performance of the system

Concerning *in silico* modelling:

- A series of data have been provided and will be provided to elaborate kinetic models for transport in both cell lines. This will allow the exploration of the predictive utility of new *in vitro* models;
- Several indications have been obtained and will be provided on identification of gaps and needed refinement in elab-

Figure 5. Hierarchical clustering of filtered normalised expression data obtained from two-colour microarray experiment, where the Caco-2 LD differentiated cells are screened versus the Caco-2 LD cycling cells. Log_2 of absolute expression values are shown. The set used for clustering is composed by the 1183 genes that show an average ratio differentiated/cycling greater than 2.0 or smaller than 1/2.0 (in the linear scale) in normalised data, where the normalisation procedure included both the Lowess and dye-swap methods. The 8 columns correspond to the Cy3 and Cy5 separate Lowess normalised channels for each of the 4 arrays; 2+2 biological replicates and the corresponding technical 2+2 dye-swapped replicates (one per sample) are shown. The column headers have the following meaning: C, cycling cells; D, differentiated cells, 1 and 2 refers to the biological replicate samples; Cy5 and Cy3, refers to the cyanine label (either cyanine 5 or cyanine 3). It is clear from the sample tree on the top of the diagramme that the chosen gene set is very effective in discriminating cycling and differentiated cells, since the biological replicates cluster together, cycling samples on the left and differentiated ones on the right, moreover within each of these two groups the technical replicates also cluster together.

orating ADME and PBPK from *in vitro* cellular models.

Problems

- The production of differentiated hepatocytes through the transfection of specific factors with the lentiviruses. While the technical approach has been shown effective on Hela cells, it has been proven very difficult on the hepatoma cells. Thus, this goal has been only partially achieved.
- Also concerning the *in silico* modelling, the initial expectation has not been fully met: at the very beginning, a difficult reciprocal understanding of the possible approaches was faced by biologists and *in silico* researches. This has been successfully overcome, but the production of useful data for modelling has been delayed according to the optimisation of the procedures.

Solution(s)

The development of a variety of viable and differentiated hepatocytes besides those developed in this project can be useful, allowing a choice between different cellular models: the progress made in this project for what concerns the stem cell differentiation and the transfection with specific factors have paved the way for future more successful developments.

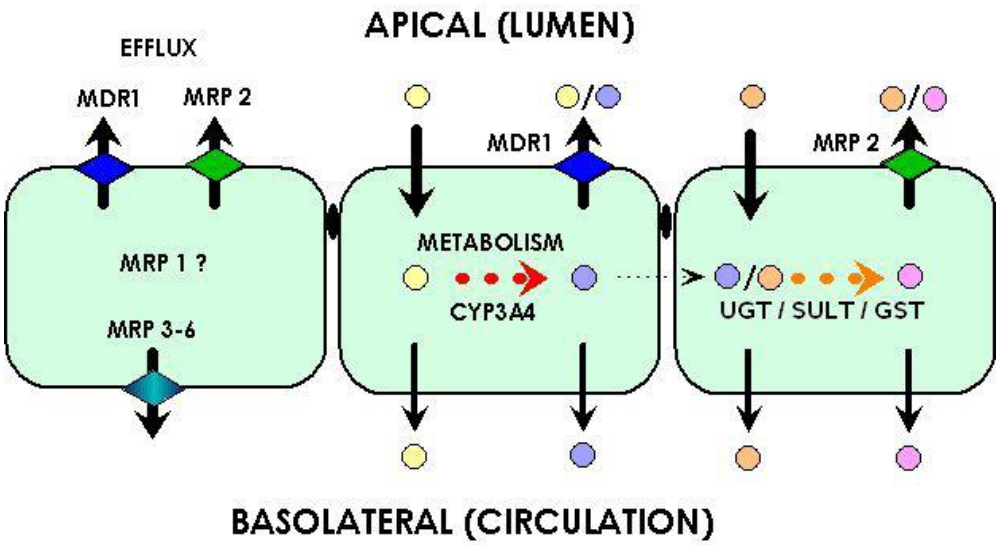


Figure 6. Potential patterns of interplay between the most important drug efflux and metabolism processes observed during the Caco-2 permeability studies. Localisation of the efflux proteins are presented as diamonds and drug molecules as circles; parent drugs in yellow and orange, phase I metabolites 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).

Concerning the *in silico* models, several difficulties have been overcome; on the other hand, the experience has allowed producing a series of indications to optimise future studies on the elaboration of predictive models from *in vitro* cultured human cells.

Next Steps

- Metabolic performance and the cytotoxicity testing, on the optimised hepatocytes and enterocytes models.
- *In silico* modelling, from data on absorption and transport.
- Co-culturing of hepatocytes and enterocytes, into the direction of more complex *in vitro* systems.
- Genomic comparison of normal small intestine with the optimised Caco-2 cell line.

Publications

The updated list of publication is available on the website *http://www.liintop.cnr.it* under "dissemination".

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ARTEMIS

In vitro tissue for replacement of transgenic animals with memory/learning deficiencies

Contract number:	LSHM-CT-2007-037862
Project type:	SME- Specific Targeted Research Project (FP6)
EC contribution:	€ 1 984 900
Starting date:	1 March 2007
Duration:	45 months
Project website:	**http://www.artemisproject.eu**

Background

The goal of the project is to develop and evaluate an *in vitro* system composed of a network of synaptically-interconnected neurons. The system could replace the memory/learning tests of drugs and neurotoxic compounds that are performed in animals. It could also be used as a memory/learning disease model system to replace studies performed in transgenic animals.

The neurons are generated by mouse embryonic stem (ES) cells are placed in a hydrogel biomaterial to develop their synapses in three dimensions (3D). The hydrogel is placed inside a perfusion bioreactor for long-term cultures needed for the development of the synaptic network. The synaptic network develops under electrical stimulation so that its final topology is stimulus-specific. This topology corresponds to the "memory" of the stimulus that the system has acquired. This "memory" is reflected to the features of the signal with which the system responds when stimulated with the same stimulus that has been used during the network development.

In the first phase of the project, we developed and tested the components of the system and we have assembled them in an integrated system.

Neurons have been generated from ES cells using different differentiation protocols. Their properties have been checked with the expression of gene markers and global gene expression analysis. Hydrogels in which the neurons can attach and grow have

been developed and their physical properties determined and optimised for the cell growth (Chapter 1). Perfusion bioreactor systems that carry the hydrogels seeded with neurons have been installed, and their operation has been optimised to assure long-term cell viability. Microelectrode arrays have been incorporated in the bioreactors in contact with the neurons of the hydrogel (Chapter 2). The electrodes have been connected with a system for electrical stimulation and response signal amplification, noise extraction, spike detection, and data storage. New algorithms for statistical analysis of the response signal have been developed and installed in the system. The system has been checked in experiments with neurons inside hydrogels, and signals have been recorded and analysed.

In the second phase of the project, the neuronal network developing in the *in vitro* system has stimulated to acquire the memory of the stimulus and various tests have been performed. Different types of neurons were used, such as cortical neural stem cells and neurons generated from embryonic stem cells with two protocols—one that gives predominantly dopaminergic cells, and one that gives GAGAergic cells. We found that neurons more easily develop bursting activity when plated on microelectrode arrays than when inside hydrogels. The bursting activity, however, increases when the neuronal network developed inside hydrogels is stimulated. We have therefore determined the effects of stimulation on the response signal (Chapter 3). These effects will be used in the third period of the project to determine whether neurotoxicants or neuronal networks developed from cell lines with genes involved in memory/learning exhibit a different behaviour, and what parameters of the response-signal change in these cases. In addition to experimental data, we have developed a mathematical model that can predict the neuronal network electrical activity from the topology of the synaptic network.

ES cell lines with genes participating in memory/learning switched-off (transgenic cell lines) have been developed (Chapter 4), and they will be used in the third period of the project in the developed *in vitro* system to give defective (transgenic) synaptic networks, whose ability to memorise the electrical stimulus will be tested from the analysis of the recorded signals. The success of these experiments will open a new research line, replacing the use of transgenic animals for memory/learning diseases with an *in vitro* system of transgenic synaptic network (or transgenic neural tissue).

Experiments with neurotoxic compounds that influence memory will be performed in the third period of the project in the system, and their effect on the ability of a normal synaptic network to memorise electrical stimuli will be determined. The success of these experiments opens a new area for the use of the *in vitro* system, which was until now restricted to evaluate biochemical effects of neurotoxins, which however could not be extrapolated to behavioural effects referring to high-level functions as such memory and learning are.

It is envisaged that further development of the *in vitro* neuronal tissue system will incorporate such additional high-level functions besides the memory/learning, bridging the gap between *in vivo* and *in vitro*.

In the present report, we present the progress of the first and second period of the project, referring to the *in vitro* system development (first period, Chapters 1 and 2), the analysis of the effect of stimulation in the *in vitro* neuronal network developing in the system (second period, Chapter 3), and the generation of transgenic cell lines (second period, Chapter 4).

Chapter 1: Three-Dimensional Cell Growth/Differentiation

Two differentiation protocols have been tested for the neuronal differentiation of the mouse embryonic stem cell line R1 to

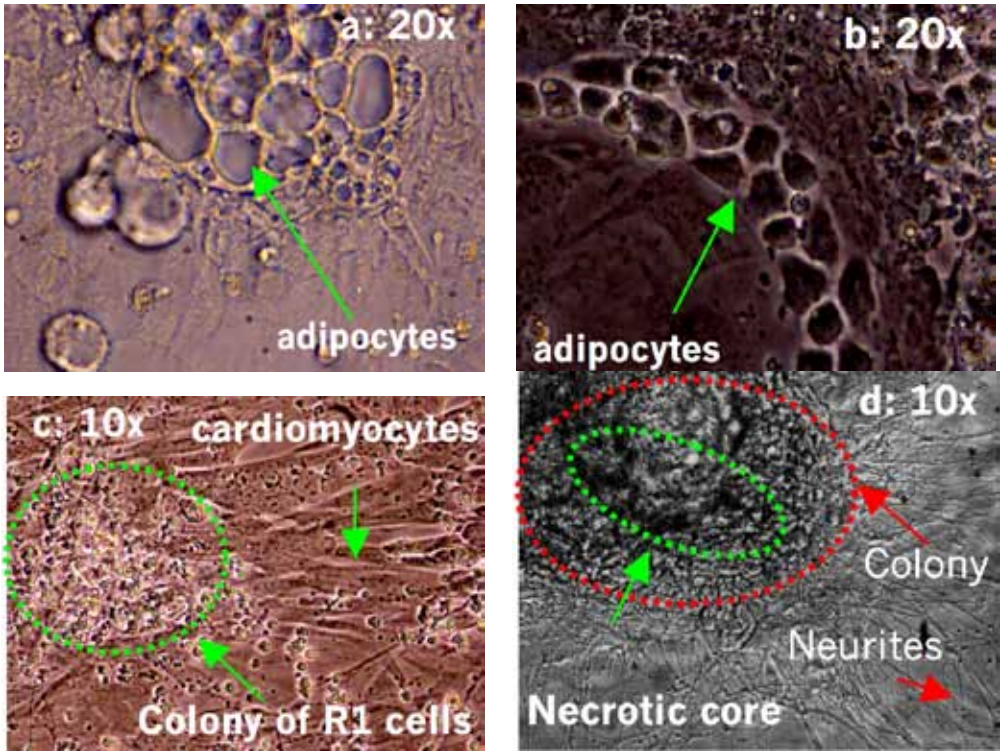


Figure 1. a, b. Adipocytes appearing during the R1 neuronal differentiation probably arising from the feeder cells. c. Cardiomyocytes appearing during the R1 neuronal differentiation. d. Differentiated R1 according to DA protocol. Axons can be seen projecting from the periphery of the colonies. A colony can be seen in the left upper part of the picture. Due to the aggregation of the cells in the colony, cells start dying when their density is high and appear darker under the microscope (necrotic cores).

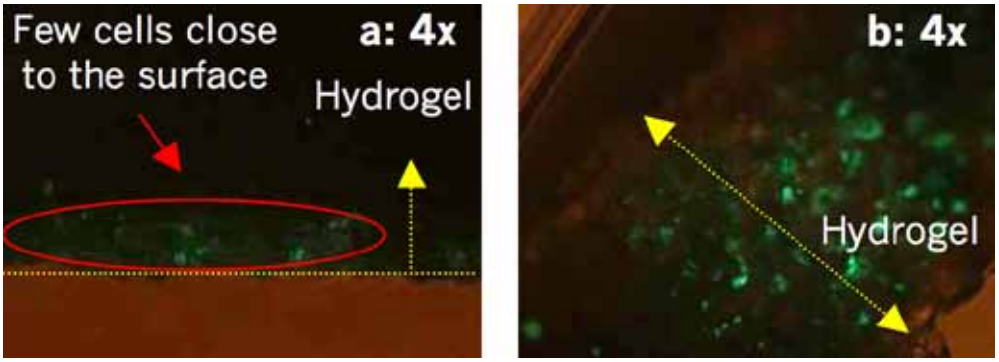


Figure 2. a. Cross-section of the hydrogel 004-04 with pore size 63-125 mm showed poor cell penetration. b. Cross section of the hydrogel 004-05 with pore size 125-250 mm shows that cells have penetrated the hydrogel. Experiments performed in static conditions. (Hydrogels: PHEMA + MADQUAT-PEGDA-004-04 and 004-05 both with 10% MADQUAT, cells: SH SY5Y).

generate neurons that will be placed inside hydrogels for the three-dimensional formation of the synaptic network. They are both modifications of the protocols described by Barberi et al., (2003). The one, denoted as DA protocol, gives predominantly dopaminergic neurons, and the other, denoted as GABA, gives predominantly GABAergic neurons. It was found that the GABA protocol has the advantage of early separation of differentiating cells from the feeder cells and the absence of adipocytes (Figure 1a, b) or cardiomyocytes (Figure 1c) that we have observed in the DA protocol. In addition, the subculture of cells at days 6 and 10 of differentiation in the GABA protocol prevents the formation of large colonies that unavoidably have necrotic cores (Figure 1d).

Several different hydrogels were prepared and tested for their ability to provide the three-dimensional support of the cell growth. It was only when the MADQUAT, a cationic monomer, was incorporated in the hydrogel, that cell attachment was observed, making the hydrogel surface more positively charged and therefore more suitable for the cells. We proceeded in optimising the hydrogel with the 10% MADQUAT. As we see in Figure 2, an increase in the pore size of the hydrogel with 10% MADQUAT significantly increased the number of cells that are retained inside the hydrogel, allowing them to penetrate and then be protected from the effect of the medium changes, having sufficient time to develop connections with the surface of the pores.

The hydrogel 004-05 with 10% MADQUAT and pore size 125-250 mm was subsequently tested with mouse cortical neural stem cells and neurons generated from R1 embryonic stem cells, giving satisfactory results as we see in Figure 3.

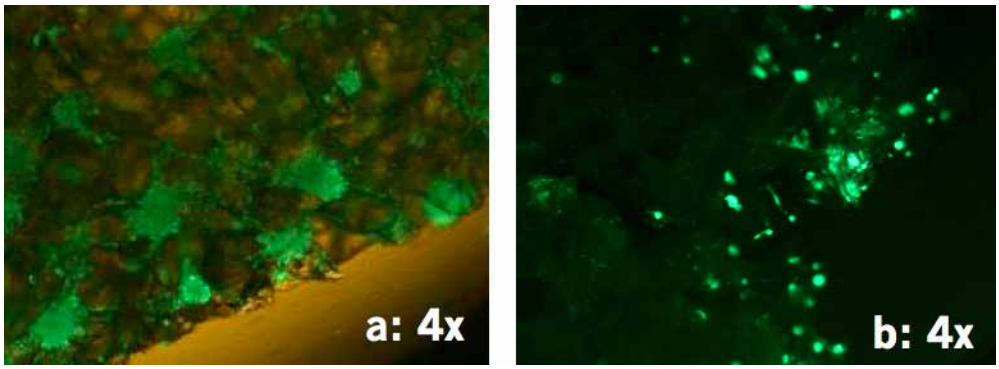


Figure 3. Hydrogel 004-05 with cortical, (a), and R1 generated neurons with the DA protocol, (b), both in static conditions.

Chapter 2: Set-Up of Bioreactor/MEA System

Set-up of Bioreactors

Two bioreactor types, Minucell-Minutissue and Epiflow, have been installed. Microelectrode arrays have been incorporated in both bioreactors. Experiments with neurons inside hydrogels placed in the bioreactors have been performed and their operation was satisfactory. After checking the operation of bioreactors further, experiments with hydrogels placed in bioreactors have been performed. In the

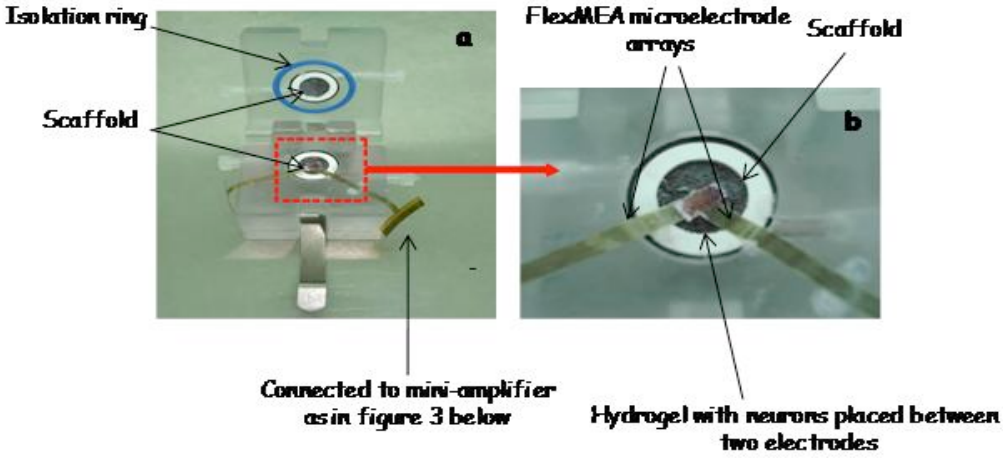


Figure 1. Minucell-Minutissue one carrier perfusion bioreactor with two FlexMEA electrodes interfacing a neuron-hydrogel matrix, a. Magnification of the chamber with the hydrogel interfaced with two electrodes, b. (Hydrogel: PHEMA-MADQUAT-PEGDA, 004-05, cells: mouse cortical neural stem cells at day 8 of differentiation).

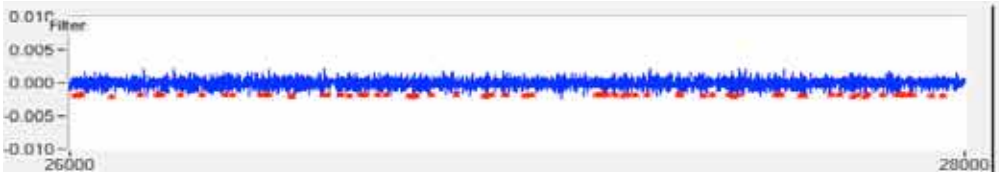


Figure 2. After removing the noise from the signal with the Butterworth algorithm, a spike detection algorithm was applied to the signal. The figures shows a part of the recorded signal with the spikes detected (red dots) by one of the microelectrodes corresponding to channel 46, one of the 32 of the array.

experiment presented below in Figures 1 and 2 as an example, a neuron-hydrogel matrix was included between two micro-electrode arrays inside a carrier (a position in the bioreactor for a hydrogel disc to be placed) of Minucell-Minutissue perfusion chamber, in which the hydrogel and electrode contact was fixed with two highly porous Cytomatrix© scaffolds, exerting a pressure, as it is seen below.

After several days of differentiation, the electrodes of the bioreactor that were connected to the MEA electrical hardware recorded the neurons electrical activity, as it seen above in Figure 2.

In the example presented in Figures 3 and 4 (below and overpage), neurons generated by R1 embryonic stem cells at day 24 of differentiation were seeded in the hy-

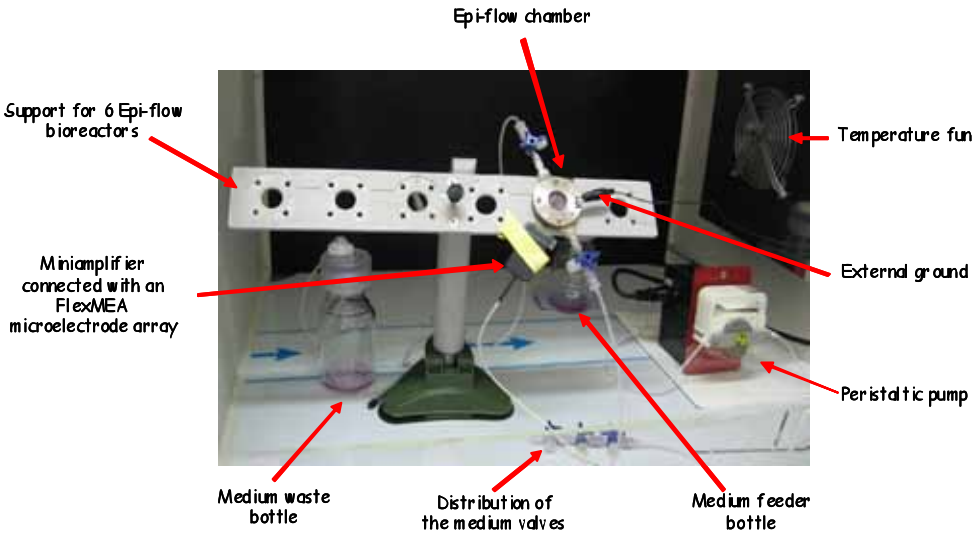


Figure 3. The Epiflow perfusion bioreactor placed in a hood that keeps the temperature at 37°C. Alternatively, the bioreactor could be placed inside the incubator. The cells were at day 27 of differentiation (2 days in the bioreactor) when signals were recorded as the one presented in Figure 4.

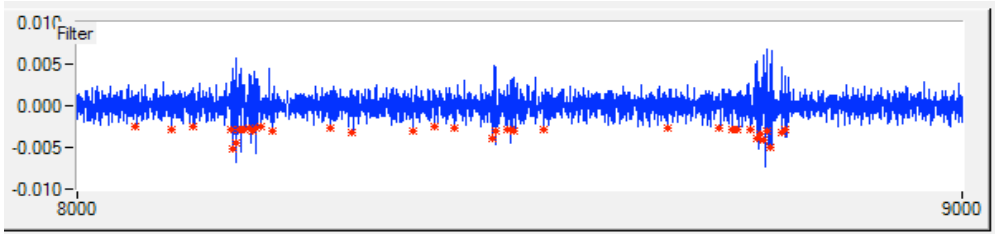


Figure 4. Spikes detected at day 27 of differentiation of R1 embryonic stem cells inside the hydrogel in the Epiflow bioreactor by the channel (microelectrode) 21. Consecutive spikes, bursts appear.

drogel that was placed inside the Epiflow perfusion chamber. In this case, two discs of the same hydrogel were used to fix the contact of the hydrogel with the electrodes, as seen in the figure below (Cytomatrix© was not used as support because it is thick compared to the thin chamber of Epiflow).

Installation of the MEA System

The FlexMEA microelectrode array, recently released in the market by Multichannel Systems, DE, which has 32 recording microelectrodes, has been selected because it is thin and it can be easily fit into the bioreactors (Figure 5). This choice determined consequently the hardware that has been installed for the signal recording. The designed MEA system appears in Figure 6 and in its implementation in Figure 7.

Another type of Minucell-Minutissue bioreactor is presented in Figure 7, which has a chamber with larger volume than the one of the Figure 1; however, both bioreactors volume is large compared to the hydrogel volume and no difference was observed in cell viability during the operation of the bioreactors.

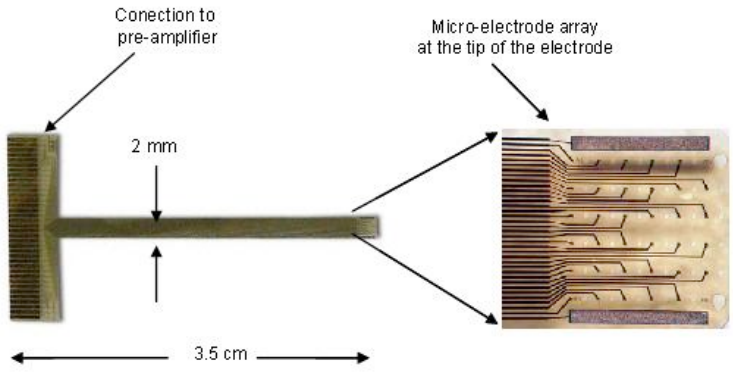


Figure 5. The FlexMEA electrode is thin and can be easily incorporated in bioreactors without the need to modify their geometry.

Tests of the Protocol of the Use of MEA with Neurons from a Cell Line Cultured Directly on the Electrode

The system has been checked with cortical cells taken from mice, which are the widely used cells with the MEA system. Tests were also performed with mouse cortical neural stem cells and neurons generated from the R1 embryonic stem cells, either directly plated on the microelectrode array or seeded inside hydrogels. In Figure 8 below, we see the spikes recorded by each one of the 32 of the microelectrodes of the array at day 3 (left) and 6 (right) of differentiation for cortical neural stem cells placed on the electrode surface. We could see that as the neurons mature, the number of recorded spikes increases. The same behaviour was observed for neurons generated from R1 embryonic stem cells.

The increase in electrical activity with the differentiation day is probably due to the increase of cell number as they multiply, to the neurons' maturation, as well as to the development of the synaptic network through which neurons activate other neu-

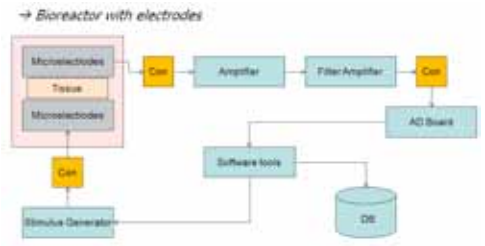


Figure 6. The MEA system architecture.

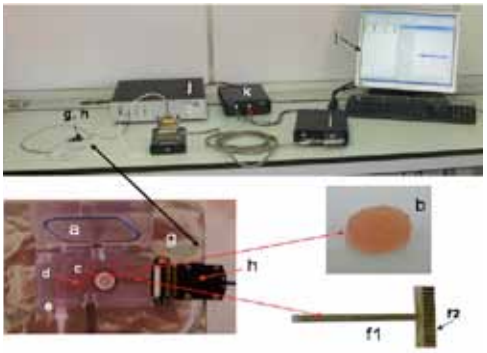


Figure 7. a. Minucell-Minutissue perfusion bioreactor with the cap containing the blue ring for isolation of the chamber open, (the bioreactor is inside the incubator during the experiment). b. Neurons-containing hydrogel. c. Hydrogel inside the bioreactor restricted by a ring. d. Medium surrounding the hydrogel perfused through e. f1. FlexMEA microelectrode array with the tip, (array), in contact with the hydrogel. g. Adapter for the connection of the electrode in side f2. h. Mini pre-amplifier. i. Filter amplifier. j. Stimulator. k. Power supply. l. Data storage and analysis.

	340	501	734	503	
515	35	716	360	239	438
302	264	509	599	567	743
489	951	521	446	439	571
636	131	275	310	428	212
	0	466	913	217	

	1148	1932	796	518	
1033	984	916	275	820	373
2786	3413	2824	1198	2102	2449
1062	2852	1319	1294	1432	1544
845	1252	1216	1113	1268	996
	0	1070	1027	1106	

Figure 8. Maturation of neurons is translated in higher electrical activity as determined by the number of the detected spikes. Mouse cortical neural stem cells at day 3 and 6 of differentiation (left and right, respectively).

rons and vice-versa keeping them in an activated state. When the cell density on the electrodes increases, the number of detected spikes also increases because more neurons are in contact with the microelectrode tips of the array (see the relative size of the microelectrode tip and of the cell in Figure 9c).

In Vitro System Evaluation

Several tests have been performed to characterise the generated neurons. Staining with GFAP in neurons generated from R1 embryonic stem cells detected glial cells (Figure 10, overpage).

We have detected by gene expression analysis performed at days 6, 8, 10, and 12 the expression of Olig-1, which is a transcription factor with a restricted expression seen in the oligodendrocyte lineage. Olig-1 is expressed early and appears specifically required for the development and maturation of oligodendrocytes. Its detected expression signifies that the protocol we have used is able to give oligodendrocytes.

Synaptophysin is a glycoprotein present in the membrane of neuronal presynaptic vesicles, and it indicates connections between neurons. Immunofluerescence staining was performed with anti-synaptophysin antibody, showing that this protein is expressed and therefore the neurons

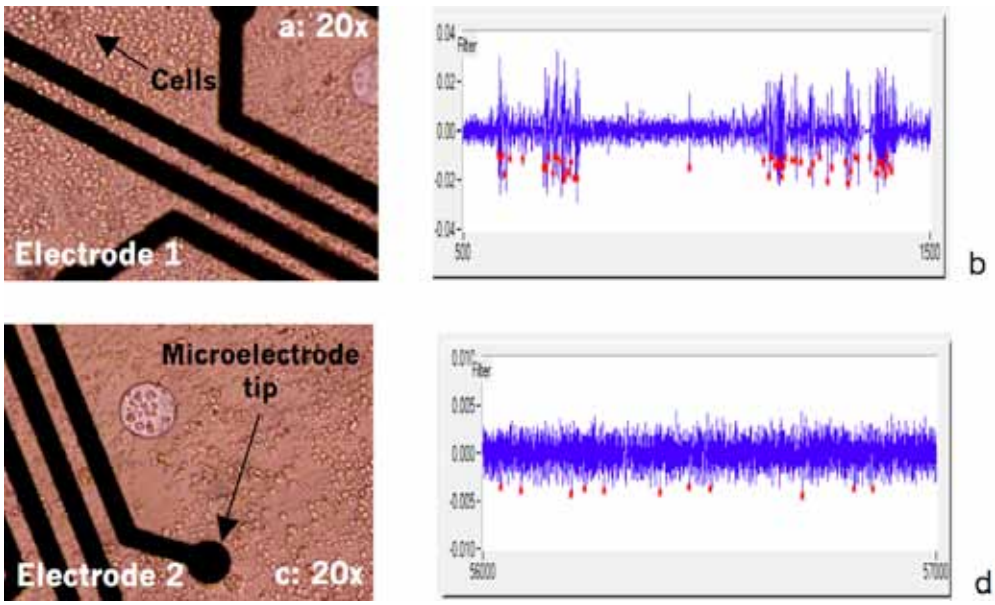


Figure 9. The second recording (channel 53) d, which corresponds to the electrode 2, has fewer spikes than the first one (channel 54) b, which corresponds to electrode 1. This is in accordance to the pictures of the electrodes plated with cells that show much more cells in the electrode 1, a, than in the electrode 2, c. This means that there are fewer neurons on or close to the tips of the microelectrodes of the electrode 1.

have developed functional synapses (Figure 11). Its expression starts as early as at day 14 and continues at least until day 44 of differentiation.

The generated neurons were also positive to synapsin, one of the major phosphoproteins in nerve terminals, which is implicated in synaptogenesis and the

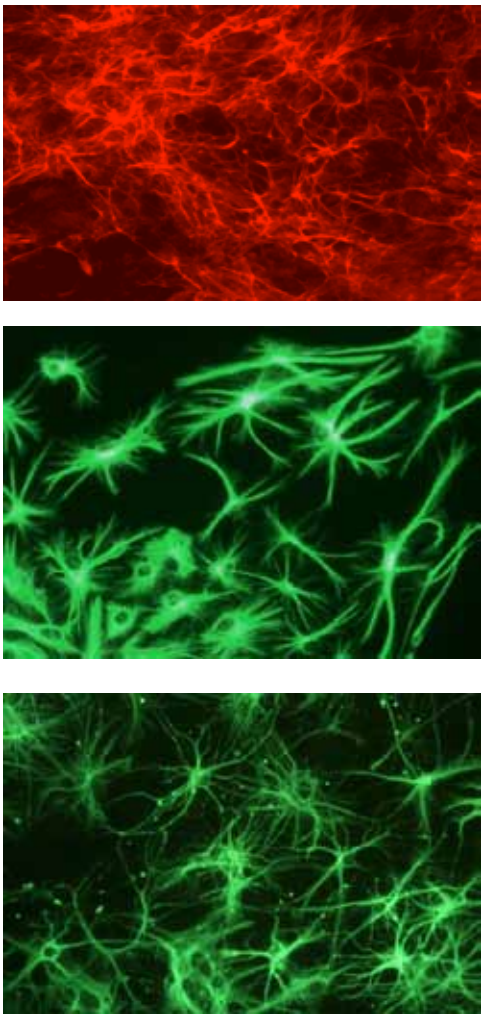


Figure 10. Immunofluorescence staining with anti-mouse GFAP at days 14 (top), 31 (middle) and 44 (bottom), of differentiation, the difference in the colour is due to the use of different secondary antibodies conjugated with different fluorescence substances.

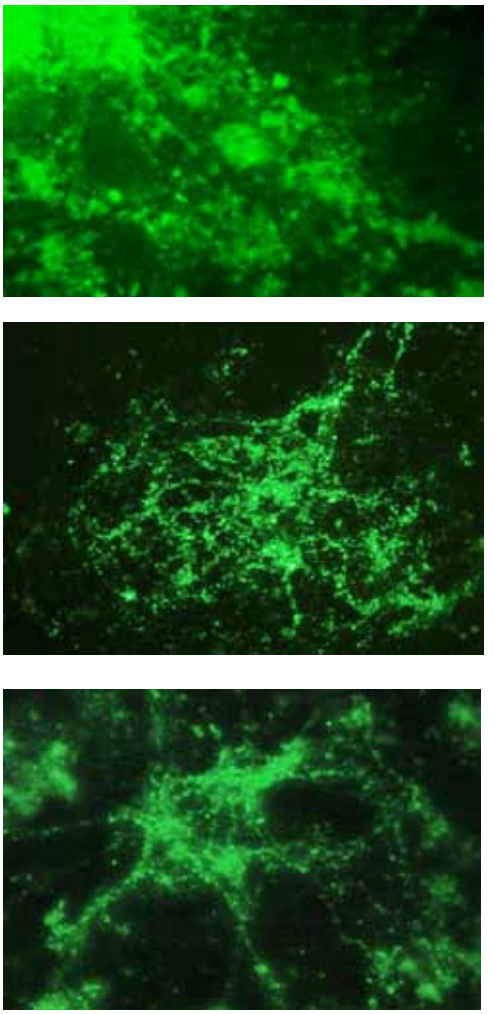


Figure 11. Immunofluorescence staining with anti-synaptophysin at days 14 (top), and 44 (middle), of differentiation for neurons generated with the DA protocol and 35 (bottom), of differentiation for neurons generated with the GABA protocol.

modulation of neurotransmitter release and plays a role in regulation of axonogenesis and synaptogenesis. The expression of the receptors for NMDA and GABA (the major inhibitory neurotransmitter in the vertebrate brain) was confirmed by gene expression analysis.

We may therefore say that we have evidence for the functional maturation of neurons, either at the neuron level with the expression of the receptors for NMDA (important because it is involved in the phenomenon of long-term-potentiation, LTP) and GABA, or at the network level with the formation of functional synapses. Further, this maturation is seen in the experiments with electrodes where signals have been recorded.

Chapter 3: Electrical Activity of Neurons

A. Spontaneous Activity

We had decided from the first period of the project that we will use the GABA protocol of differentiation because it gives a more pure population of neurons without adipocytes and cardiomyocites as the DA protocol, and with fewer necrotic cores inside the colonies. Neurons are initially differentiating in well plates where the contact with the feeder cells that provide growth factors is possible. They are transferred to hydrogels and left for 48 hours in static conditions for the cell attachment to be completed. Then the neuron-containing hydrogel is transferred to the bioreactor and is interfaced with microelectrode arrays. We have recorded the activity of the synaptic network along the differentiation days, as it is seen in the figure below expressed as the total number of spikes detected by all the microelectrodes, 32, of the array (MFR).

A peak of the activity is presented by both new experiments (day 18 for experiment 1 and day 20 for experiment 2). A more detailed examination of the recorded signals will be presented overpage. The temporal relation between the detected spikes on the microelectrode and array level determines the major type(s) of activities:

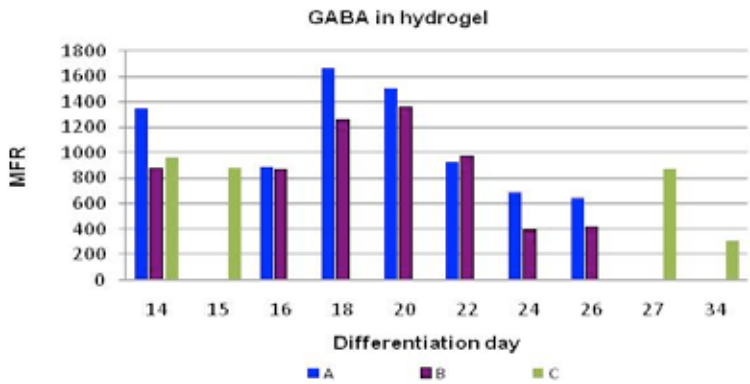


Figure 1. Electrical activity as expressed by MEF of neurons generated by embryonic stem cells, seeded inside hydrogels and interfaced with microelectrode arrays in a perfusion bioreactor system.

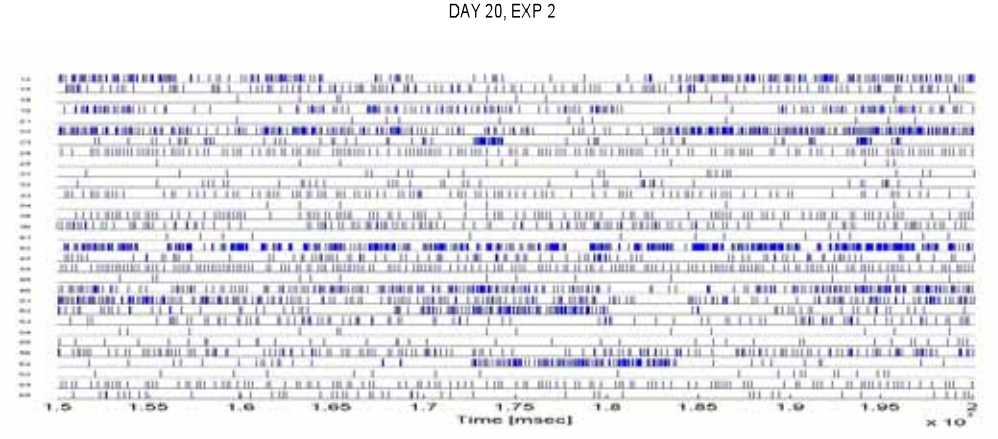


Figure 2. Raster plot of experiment 2 at the maximum activity at day 20 does not show network bursts but only bursting activity at the single microelectrode level (horizontal blue zones of intense spiking activity areas). (When a spike is detected in a microelectrode at some time point, a short line is placed and the (x, y) plane of the raster plot).

1. Asynchronous firing. Neurons fire spikes in an uncoordinated way.

2. Bursts. This is the most common pattern of activity in primary cortical neurons, where bursts (i.e., several spikes close to each other) of intense activity are separated by long periods of near-quiescence (very few sporadic spikes) activity.

There are two types of bursts:

a) *Single microelectrode bursts.* Short bursts of intense activity in single microelectrodes of the microelectrode array are separated by long periods of near-quiescence (inter-burst intervals) with sporadic spikes detected. "Raster plot" diagrammes are used for the visualization of the network activity. The x-axis is the time and the y-axis the number of the microelectrode of the array (Figure 2 above). Microelectrode bursts in such diagrammes appear as small vertical lines horizontally (same microelectrode) arranged in very close distance from each other (blue horizontal zones in Figure 2). The appearance of microelectrode bursts signifies that neurons in the same microelectrodes fire in short delays between them, with the one activating the other so that the activity will be spread to all neurons on a microelectrode. The periods of sporadic firing between microelectrode bursts, in which only few spokes are detected (few horizontal lines in Figure 2 between blue horizontal zones) arise from few individual neurons, with most of them casing firing in order to replenish their resources and be ready for the next burst.

b) *Network bursts.* The network bursts are composed of single microelectrode bursts that happen simultaneously for

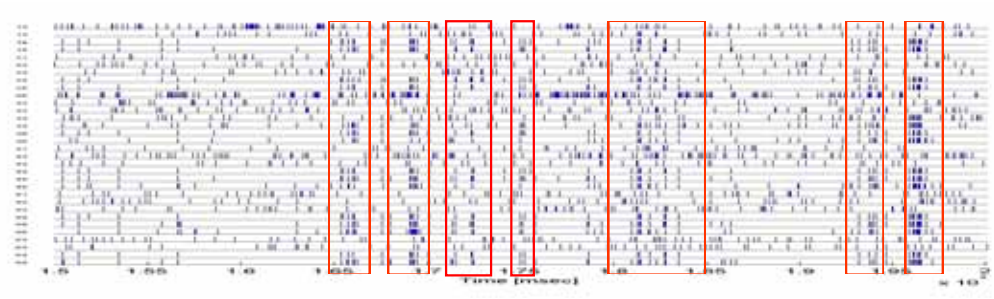
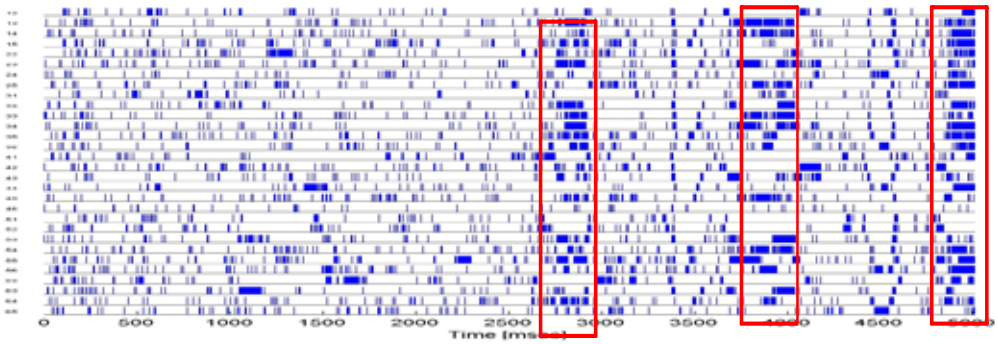


Figure 3. Network bursts appearing in day 16 of differentiation probably due to horizontal growth, and the interconnections developed among the neurons on the hydrogel surface in contact with the electrode surface that take place before the colonies will grow towards the inside of hydrogels penetrating the pores.

Figure 4. Network bursts due to the horizontal synaptic interconnections of neurons on the hydrogel surface re-appear in later differentiation days.



several different or all the microelectrodes of the array (globally synchronized bursts). In the raster plot diagramme, such activity is represented by vertical zones, i.e., microelectrode bursts (horizontal closely occurring spikes) arranged vertically (which means at the same time).

As we see in the figures above, there is no network bursting activity (no vertical blue zones) in the days of maximum activity of Figure 2 overpage, but several microelectrode bursts (horizontal blue zones).

The network bursting activity requires a synchronisation across the whole network, which means that a global synaptic network has been developed. While this is easy when neurons are cultured in two-dimensional supports, it is difficult inside the hydrogels because the extension of the several colonies residing inside the pores of the hydrogel is restricted by the pore walls, so that they could not give larger colonies covering at the same time many microelectrodes with their collision. Then we expect that initially we could see some bursting activity arising from the

horizontal connection of neurons that are in contact with the electrode. And indeed as we see in Figure 3, weak network bursts appear earlier than the maximum activity day (which is mostly due to the growth of colonies inside the pores).

As cells however grow inside the pores, the microelectrode bursts (spikes detected by each microelectrode in contact with a colony) dominate the activity because there is large available space for cell growth inside the pores so that the synchronisation of the neurons' activity for a colony growing inside a pore is easy facilitated also by the neurons maturation along the time. If such is the case, we should expect that when this intense activity from the colonies inside the pores (seen as microelectrode bursts) drops, probably with main reason the cell death due to the lack of nutrients and oxygen, the activity of the network on the surface should appear again. And indeed if we examine the raster plots for later differentiation days when the activity is low (Figure 4), we could detect again network bursts as it is seen in the figure overpage.

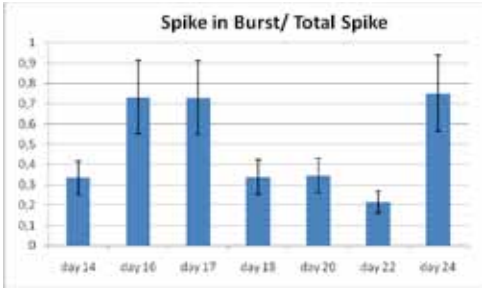


Figure 5. The evolution along of differentiation days of the ratio of spike in bursts to total spikes shows an alteration of spiking and bursting activity. (Error bars are the standard deviations from the recorded experiments.)

The above considerations for the evolution of the activity of the network developing in hydrogels can be supported by quantitative analysis of the recorded signals (besides the qualitative visual one based on raster plots). In Figure 5 below, the ratio of spikes in bursts to total spikes, which is an indication of the typology of network behaviour (when it is mainly spiky the value goes towards zero, when it is mainly bursts the values goes toward one), the ratio changes ranging from (sporadic) spiking activity (day 14) to bursting activity (days 16-17), then spiking activity (days 18-22), and finally bursting activity again.

B. Stimulation

What is expected with the stimulation of a neuronal network is to show the plasticity of the synaptic network that takes place with changes in the extension or retraction of neurites, in the formation or elimination of synapses, or in the strengthening or weakening of existing synapses, all these induced with the stimulation. In preliminary experiments we have determined the voltage and frequency of stimulation pulses with high chance to influence the network activity through changes in the synapses. These data lead to the design of the cycle of stimulation applied for each day, as it is seen in Figure 6 (overpaage). The cycle is as follows: A train of pulses was given in 4 microelectrodes simultaneously. After 15 minutes, the train was applied in 4 other microelectrodes (15 min needed for the manual change of electric

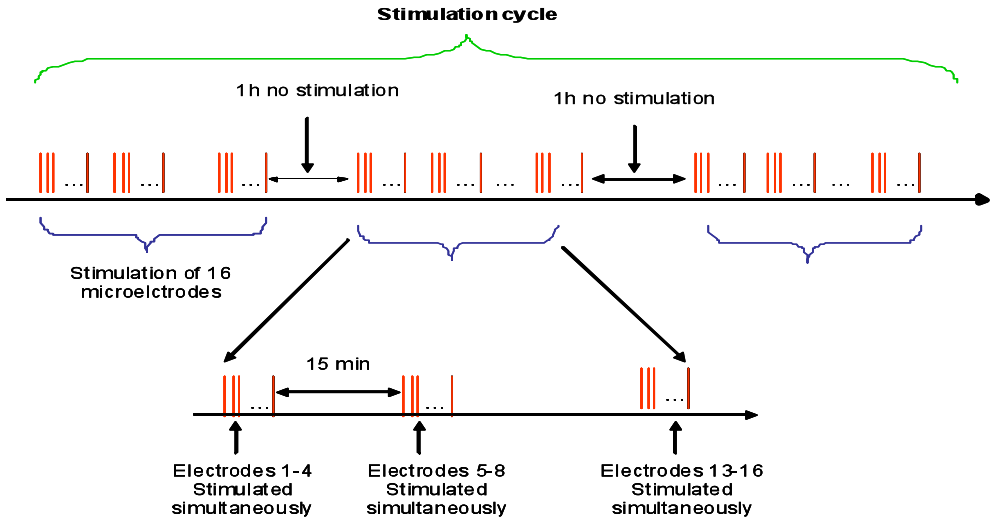


Figure 6. Stimulation cycle repeated three times per day. It consists of 3 stimulation of 16 microelectrodes in sets of 4.

wiring of stimulation cables), then other 4, and finally other 4 (i.e., 16 microelectrodes were stimulated in 45 min). After a resting period of 1 hour between cycles of trains, the cycles were repeated to give a final 3 cycles lasting around 4 hours, which makes the stimulation cycle. This stimulation cycle was repeated once every day starting from day 16 in order to allow some time for the neurons to mature and to start developing connections from day 11 of platting (the stimulation had no long lasting effect until day 16 as we have seen in the previous experiment). The stimulation ended at day 20, because in later days the long lasting effect was found negative. For the days 14, 16, 22, 24 and 26 without stimulation, the activity was recorded.

Two experiments were performed with this stimulation protocol and the MFRs were calculated, as seen in the figure below. If we compare the MFRs of the stimulation experiments with those of non-stimulation

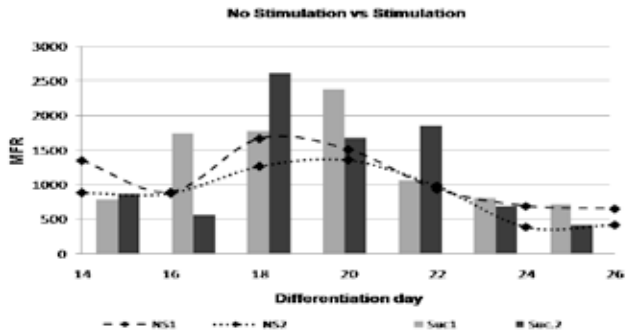


Figure 7. Stimulation increases the MFR. (Doted line the average of MFR of non-stimulated experiments and dashed line the average of two stimulation experiments).

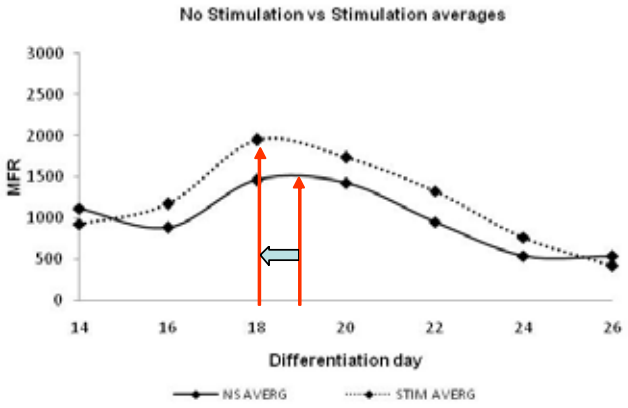


Figure 8. Stimulation increases the MFR but also reaches the maximum MFR earlier.

ones, we could see that generally stimulation increases the MFR (Figure 7; the dashed line represents the average MFR of the two stimulation experiments, and the dotted line the average of non-stimulated experiments).

An interesting observation is that the stimulation shifts the maximum MFR in earlier time as we see in Figure 8 above

In Figure 9 (overpage), we compare the raster plots of the two successful stimulation experiments with the non-stimulation one that shows the more organised activity in bursts after stimulation. As we observe, the bursting activity in the stimulation experiment 1 (first raw) starts at day 16 and

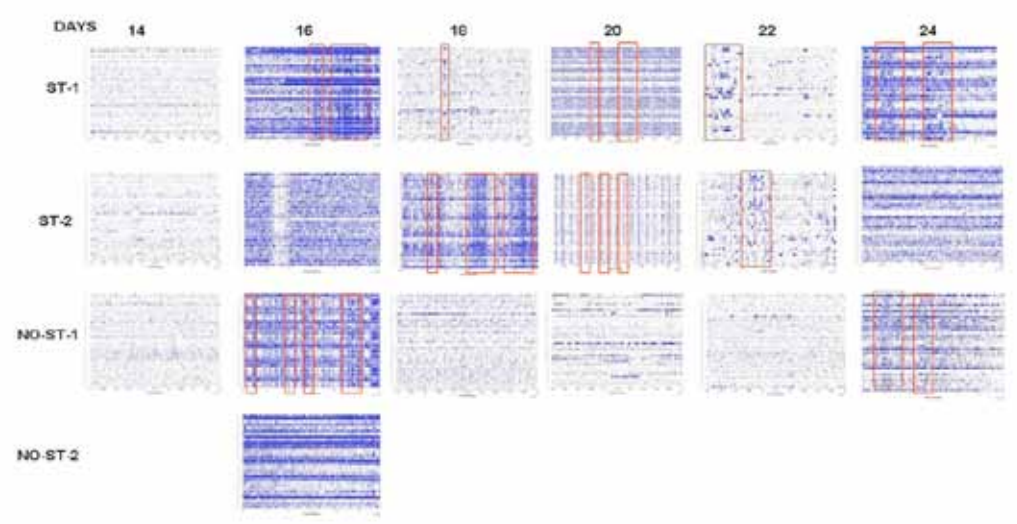


Figure 9. Raster plots of stimulated and non-stimulated networks show a longer time bursting activity in stimulated networks (stimulation from day 16 to 20).

continuous until the day 24 (though weak), with periods of weak bursting (day 18). (From the many network bursts of day 20 only few are shown.) In the stimulation experiment 2 (second raw), the bursting period is from day 18 to 22. (From the many thin network bursts of day 20 only few are shown.) In the non-stimulation experiments (third raw), there is a long period from day 18 to 22 without bursts that appear later in day 24, however weak. The bursting activity starts also early at day 16 (as in stimulation experiments since there is no stimulation until the day 16), though in some experiments we did not observe bursting (fourth raw). The differences between stimulated and non-simulated network after the day 20 are due to previous days of stimulation because the stimulation stops at day 20.

The quantification of ratio of bursting to spiking activity (Figure 10) also confirms the qualitative picture from the raster plots. For example, the highest bursting activity (almost 90% of the spikes belong to bursts) appears at day 20 in the first stimulation experiment, as we also see in the raster plot. For the second stimulation experiment, high bursting activity can be

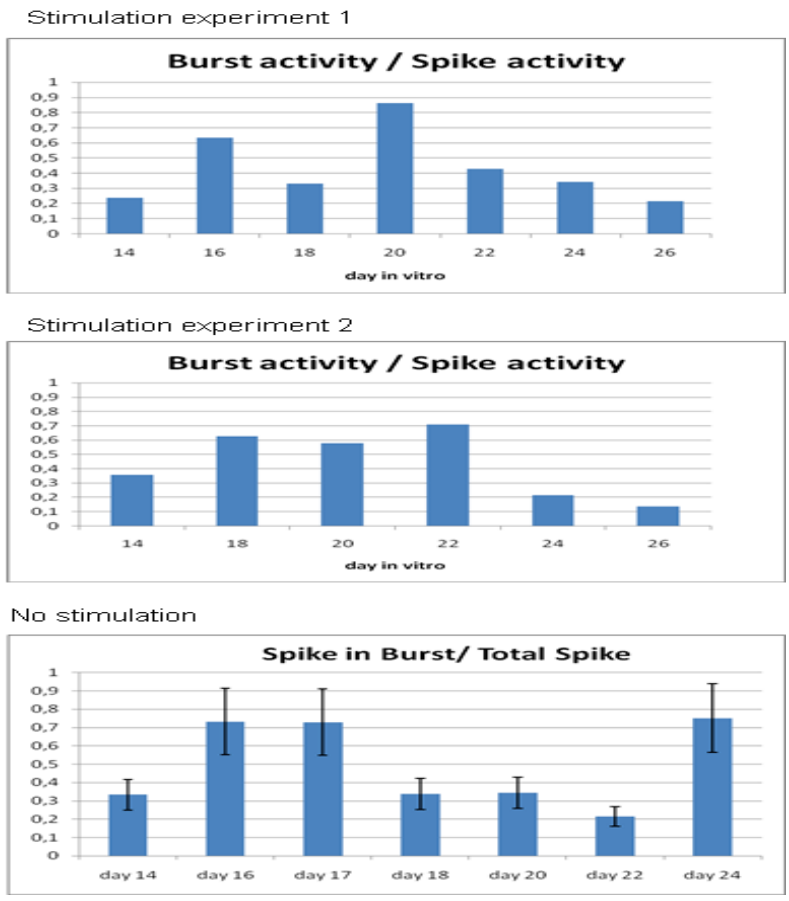


Figure 10. The ratio of spikes in bursts to total spikes in the two stimulation experiments (two upper graphs) and the non-stimulation experiments (lower graph).

seen in days 18, 20 and 22 (almost 70% of the spikes belong to bursts). The important observation is that the bursting behaviour of days 18, 20, and 22 for the stimulation experiments is (first two upper graphs of Figure 10, overpage) are higher than this for the same days of differentiation in the non-stimulation experiments.

Below we analyse the reasons why changes in the network activity have been observed in our system, examining the available literature. In general terms, the most important factor is that the network is stimulated while still under development, and therefore the stimulus has a chance to direct the synapse formation in accordance with similar studies from literature. For example, Vajda et al. (2008) mentioned that in a fully developed network, it is not easy to influence the activity. The authors mentioned that the stimulus can follow different synaptic pathways in the same topology synaptic network, which has been fixed since the synaptic connections already took place. This topology of active synapses is a sub-set of the whole network, and it is called attractor state because it is stabilised in time. When the network is temporarily disturbed by the stimulation, a transition for one attractor state to another takes place, i.e., the pathways of activations from neuron to neuron changes inside the existing stable synaptic network. In this case, therefore, there is no change in the synaptic network topology, but the spontaneous activity and stimulation follow different activation pathways. However, it is different with neurons in immature stage and under continuous development of their synaptic network, as in our case. In this case, there is no fully developed network that will allow the stimulation to just use different pathways from the already existing. The pathways are formed together under the stimulation and are stimulation-specific.

Another difference of cultures of neurons from ES cells used here is their weak bursting activity, which facilitates the changes in the synaptic network by stimulation. Such cultures do not have the high bursting activity of primary cortical neurons. Bursting is a behaviour that prevents the stimulus from influencing the network behaviour. If the culture exhibits spontaneous bursts synchronised across the whole network, there will be no substantial measurable effect of stimulation on the network activity (Zho and Poo, 2004). A high number of bursts in non-stimulated networks saturates the synapses so they could not be affected by stimulation (Corner et al., 2002). Corner et al. for example proposed that burst quieting by distributed electrical stimulation is needed to observe substantial differences in activity after stimulation. This has been shown experimentally. Bonifazi et al. (2005) have shown that blockage of excitatory synaptic pathways mediated by NMDA receptors that are responsible for the bursting activity improved the mutual information between the evoked response and stimulus properties. Another work that supports the connection of bursting with the lack of the stimulus effect is the one of Li et al. (2007). The authors showed that before stimulation, the firing pattern of spontaneous activities was random, including small bursts and single

spikes, as in the early stage of the cortical networks development *in vitro*. After stimulation, the network activity became organised in bursts sequences, as we have observed in our experiments. The effect of stimulation lasted for several hours. The authors reported that the effects of stimulation can be maintained for several hours and attributed this to long-term potentiation (LTP) at the level of neuronal network. Another reason why stimulation can more easily have an effect in neurons generated from ES cells has to do with LTP. This is an important cellular mechanism of synaptic modification that results in the strengthening of synapses (Bliss and Lomo, 1973). However, strong synapses, such as primary cortical neurons in a fully developed network, undergo relatively less LTP than weak synapses, such as those in a developing network (Debanne et al., 1996).

Another factor that played a role in our cultures was the use of retinoic during the stimulation. Retinoic induces the formation of synaptic vehicles (Sarkanen et al., 2007), increases the proportion of cells having neuritis, and increases the extent of branching (Simpson et al., 2001). Examples from the literature show that in such networks, the stimulation can have permanent effects because it influences the way the network develops, while in the primary cortical cell cultures it is already developed when it is stimulated. For example, Uroukov and Bull (2008) cultured spheroids—cell aggregates—of cells isolated from the neuroepithelial tissue of hen eggs at E7 embryonic stage on microelectrode arrays, and they stimulated them repeatedly 7 times for periods of 10 minutes each, with 5 minutes resting between stimulations. They found that the electrical activity of the spheroids (spikes per time) changes during the time of 7 stimulations. At the beginning of the culture (i.e., just-prepared spheroids), they observed a small increase of the activity. For spheroids of later stage (i.e., more time on microelectrodes), they observed a substantial increase of the activity—almost 2.5 times more than the one before stimulation, while for older spheroids a decrease of the activity after stimulation.

C. Mathematical Model of Neuronal Networks Activity

For the correlation of signal and synaptic network features, we have developed a mathematical model that has been published (Golstev et al., 2010). The model can be used with an input of the network activity that we have analysed in stimulated and non-stimulated networks, and from this it can propose network topologies that should have this activity. The network parameters that were included in the model are: fractions of excitatory and inhibitory neurons; degree distributions of excitatory and inhibitory neurons (excitatory-excitatory, excitatory-inhibitory, inhibitory-excitatory, and inhibitory-inhibitory synaptic links); efficiencies of these synapses; spiking behaviour of inhibitory and excitatory neurons (i.e., the dependence of firing rates on inputs); activation thresholds and membrane time constants of inhibitory and excitatory neurons; the rate of spontaneous activity of excitatory

and inhibitory neurons; a modular structure of the network (i.e., sizes of modules or clusters); and synaptic connections between clusters. Using these structural and dynamic parameters, one can simulate dynamics and study a burst activity in neural networks and compare these with the experimental data for living neural networks. This comparison will permit us to understand the role of the network structure and dynamic properties of individual neurons in dynamics of neural networks. In our preliminary simulations and analytical calculations, we have analysed neural dynamics and bursts at various values of the parameters for neural networks having the structure of classical random graph and scale-free graphs.

We expect, therefore, that such a model will give important information proposing network structures that have been observed in our experiments of network activity. Some issues of particular interest include the following:

1. Behaviour of the network in variations of the network topology. It is important to examine the difference in the expected activity between uniform networks as the ones with cortical primary cells regularly used with microelectrode arrays and our networks, either in 2D on the electrode surface or 3D in the hydrogels that grow developing colonies of high cell density, and interconnections that are then connected between them with geometrical restrictions that do not exist in primary cortical 2D cultures.
2. Network activity in the presence of only weakly excitatory (GABAergic that appear first) or excitatory and inhibitory synapses (later when GABAergic become inhibitory and glutamatergic appear) with variation of their relative percentage. Our cells are GABAergic weakly excitatory at the beginning, and later become GABAergic inhibitory with the simultaneous appearance of glutamatergic excitatory. We could then check which factor is decisive for the network activity and the synchronisation we observed in our experiments.
3. Behaviour of the network with stimulation will be studied in a layered structure that will simulate the extension of the network in the third dimension inside the hydrogel. The stimulation will first influence the first layer, from which successive activations to other layers, as inside each layer will take place. We could then get information for the structural parameters of the 3D network that are responsible for the behaviour we observed in stimulated networks.
4. Changes in the network topology under external stimulation (functional topology of signal transmission pathways superimposed on the physical topology). In this case, we could estimate the topology changes we expect with stimulation at different stages in which the network topology of the non-stimulated network changes according to the general aspect of cluster formation, intra-cluster connections development, and inter-cluster connections development. We could then confirm our experimental obser-

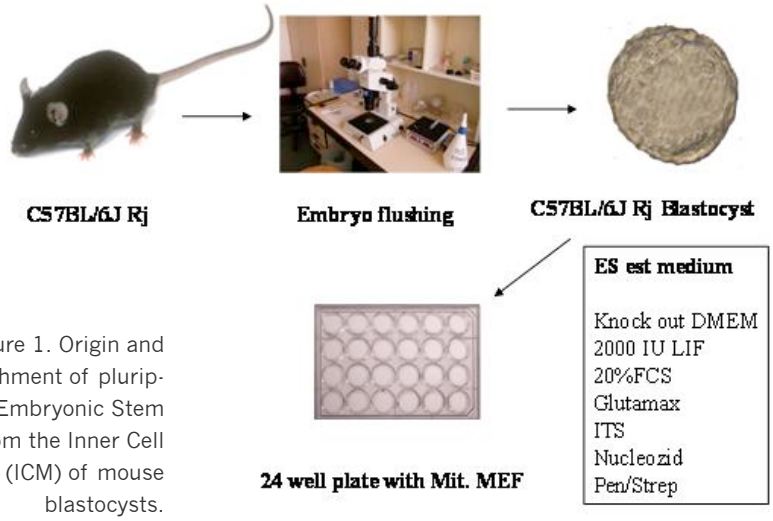


Figure 1. Origin and Establishment of pluripotent Embryonic Stem Lines from the Inner Cell Mass (ICM) of mouse blastocysts.

vations for the effect of stimulation at various stages of the network development.

Chapter 4: Transgenics & Neurotoxicity

The final goal of the project is to evaluate the *in vitro* system, i.e., the neurons developing their synaptic network inside a hydrogel placed in a perfusion bioreactor, and interfaced with microelectrode arrays for stimulation/recording, as an alternative to the use of memory/learning deficient transgenic animals and as integrative neurotoxicity test with "behavioural-like" (memory/learning) endpoints.

We have seen before the way to stimulate the developing *in vitro* synaptic network in order to exhibit an activity specific to the stimulus. This method has been successfully applied in the system, and stimulus-specific signal features have been indentified (different from the ones of the non-stimulated networks). In this sense, the developed *in vitro* system is complete and ready to be used with transgenic neural tissues or neurotoxins where the evaluation of the memory/learning ability of the transgenics, as well as the effects of neurotoxins in memory/learning will happen based on the signal features we found different in the stimulated network from those of the non-stimulated.

In the simplest case, we expect that memory defects either in transgenic tissues, or after exposure of the tissue to neurotoxins, will generate signals that do not have the characteristics of the stimulated normal tissue, as these have been described in the previous chapter. In such a case, we could say that the *in vitro* system can detect memory/learning defects without the need to generate the transgenic animals,

since the transgenic neural tissue made from transgenic embryonic stem cell lines can provide similar answers. By the same token, no necessity will exist to perform animal tests to find the effect of neurotoxins in memory/learning, as this can be done in the *in vitro* system with normal neuronal tissue developed from normal ES cell lines.

This chapter refers to the generation of normal or transgenic stem cell lines, and neurotoxicity tests that will be used for the evaluation of the system.

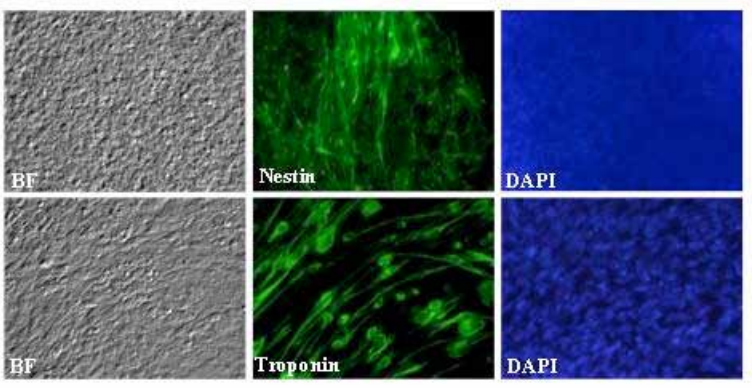
A. Establishment of ES Cell Lines from C57/BL6 Genetic Background

In mouse behavioural studies for CNS disease, animals with C57/BL6 genetic background are normally used. Studies on 129SV animals showed that the cognitive abilities and training results with such genetic background are much poorer. This situation is creating a problem when targeted genetic modifications are studied, as backcrossing to C57/BL6 is a time-consuming and costly process. C57/BL6 ES cell lines are not widely available, as this genetic background was considered non-permissive for a long time. However, recent results have shown the feasibility of establishing such lines. In order to enable a fast production of models in a desirable genetic background, we established a new ES cell line from C57/BL6 animals. The C57/BL6 ES cells will be tested in the *in vitro* system for their capability to establish functional towards memory/learning neural tissue.

A number of ES cell lines from blastocysts of SPF C57/Bl6 mouse strain have been isolated. The ES cell lines are embryo-derived stem cell lines directly isolated from the inner cell mass (ICM) of mouse blastocysts using feeder cell layer (Figure 1, left).

To characterise the new cell lines, *in vitro* pluripotency tests have been performed. Positive Oct4, Nanog, SSEA-1, and Sox2 immunofluorescent staining, positive alkaline phosphatase (AP) enzyme assay, and positive RT-PCR were observed in the newly established ES cell lines, suggesting pluripotency potential. In *in vitro* dif-

Figure 2. Representative immunostaining of one of the differentiated cell lines. Nestin and represents neural differentiation potential, while Troponin shows the cardiac differentiation potential *in vitro*.



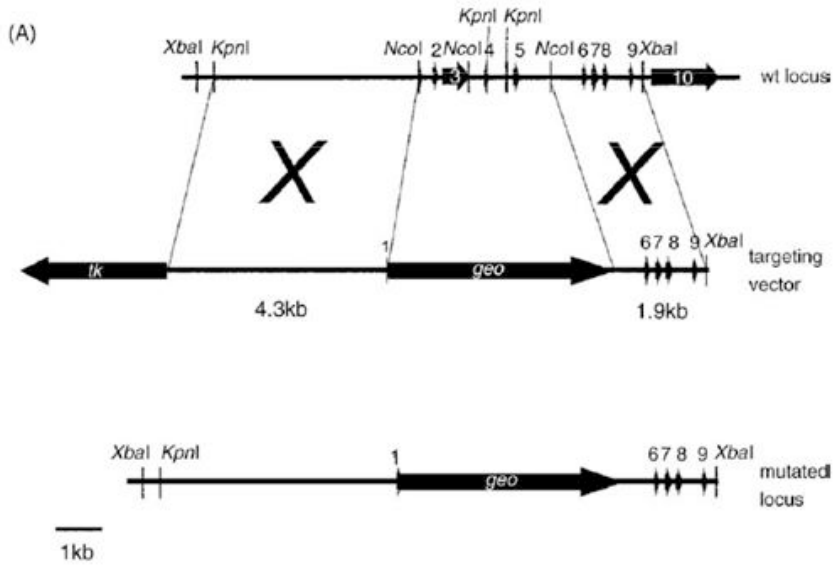


Figure 3. The figure depicts scheme for targeting of the Ric-8 locus in mice. The Ric-8 coding sequence was replaced by the GEO (G418 resistance and LacZ fusion gene). 5' homologous arm was 4.3 kb and contained intergenic and promoter area just before transcriptional start site ATG (NcoI). 3' homologous arm was 1.9 kb long and contained coding are from 6th exon.

ferentiation studies, the cell lines showed spontaneous differentiation with LIF withdrawal, and produced several cell types of all the three germ layers. When differentiation was induced with DMSO (1%) into cardiac muscle, or with RA (10-7 M) into neuronal tissue, the established cell lines could produce both cell types respectively (Figure 2).

The generated cell lines have been also successfully tested for germline transmission.

In conclusion, cell lines with C57/BL6 genetic background have been generated and tested, and are ready to be used for the generation of *in vitro* synaptic network to test the memory/learning abilities.

B. RIC-8, CCK 1,2 and WSF1 Mutants

1. Biochemical analyses of Ric-8 mutant mice

We have developed and tested knockout transgenic mice, in which the Ric-8 (AKA synembryn) gene was switched-off. These mice showed impairment of memory and learning in several tests. In the next period of the project we will develop the neural tissue of Ric-8 transgenic mice from the transgenic ES cell line in the *in vitro* system, and we will analyse the electrical signals to see if the transgenic tissue has different response to stimulus from the normal one according to the findings of Chapter 3.

Several pharmacological, biochemical, gene expression, and histochemical analysis of neural tissue of Ric-8 deficient transgenic mice have been performed, and they will compared with similar analyses of the transgenic neural tissue developing in the *in vitro* system. Ric-8 mutant mice were generated via homologous recombination (Figure 3).

We have checked the expression of Ric-8 in embryonic mice, and we found that the earliest expression was detected at E9.5 in the developing nervous system, where it was located in the crania ganglia. At day E10.5, the expression can be detected in several tissues of the developing neural system in the cranial ganglia, sympathetic trunk, dorsal root ganglia, and neural tube.

We have also checked the expression of Ric-8 in adult mice, and we found that most prominent expression was in the hippocampus (Figure 4). Thus, it is very likely that the lack of this gene in transgenic mice should induce changes in the physiology and behaviour related with memory/learning that we have observed (e.g., Ric-8 transgenic learned the initial location of platform easily, but they exhibited impaired finding of platform in reversal test).

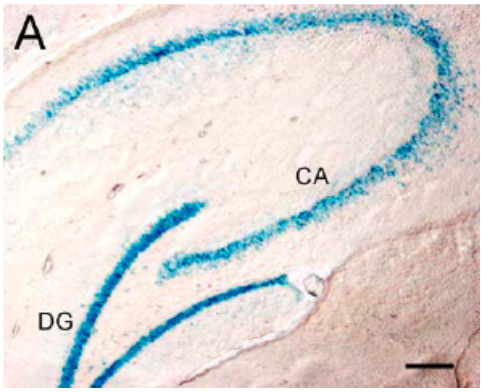


Figure 4. This figure depicts the close look on the hippocampus CA and DG regions showing strong and intensive Ric-8 expression.

We have performed biochemical analyses and found that the binding of GTP in the hippocampus and striatum is higher in wild mice than in heterozygous mutant mice, in which Ric-8 is expressed from one allele. According to literature information for the signal transduction pathways, this means that Ric-8 interacts with GDP-bound Gα subunit and stabilises the transition state of Gα proteins. The Gα proteins then activate the UNC-13 protein, which is required for vesicle fusion machinery. In few words, we have shown that the Ric-8 protein is involved in the presynaptic changes in synaptic strength, which is one of proposed mechanism for establishing and modifying behaviours or memories.

We have also performed gene expression analysis and found no difference between Ric-8 mutants and normal mice in the hippocampus. The best explanation is that there aren't very many differences in RNA levels between these two mouse lines because it is only one of the two allele genes that we had switched off in the transgenic mice. We can't use homozygous mutant mice because the die in embryonic stage.

As a result, we showed that Ric-8 is expressed during early development of mouse neurogenesis and also in the adult CNS. These data indicate that Ric-8 has

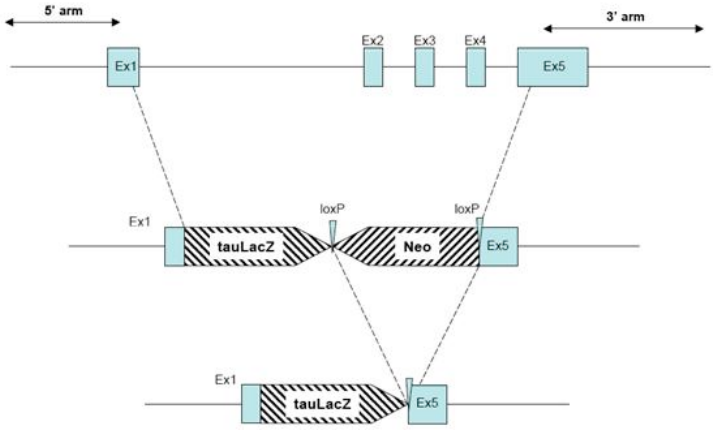


Figure 5. Strategy for the CCK2 receptor targeting in the embryonic stem cells. Neo resitace cassette is put between two loxP sites and will be removed in cell culture by transient expression of Cre-recombinase. Resulting tauLacz in frame allows later visualisation of the activity of endogenous promoter and staining of appropriate axons.

role in the development of the CNS and also in the adult brain. In the adult brain, most prominent expression was found in the hippocampus. Thus, as Ric-8 has role in the development of CNS and it is also expressed in specific regions of the brain, it is very likely that the lack of this gene should induce changes in the physiology and behaviour of mice.

The next step is to use the cell line in the *in vitro* system to generate transgenic neural tissue, and to check the electrical activity of transgenic tissue made *in vitro* from transgenic ES cells. We could correlate the electrical activity with the biochemical differences we have found, as mentioned above, and which will be also done in the *in vitro* developing transgenic neural tissue.

2. Mutant CCK1 and CCK2 cell lines

The aim was to develop of conditional ES cell lines of mutant CCK1 and 2 receptor mice to be used in the *in vitro* system for generation of *in vitro* transgenic neural tissue. The peptide cholecystokinin (CCK) acts as a neurotransmitter and is involved in learning and mnemonic processes, and the switching-off of CCK in transgenic mice leads to memory/learning impairment. However, conflicting results for the role of these receptors in memory have appeared in literature. Since the knock-out approach is susceptible to a number of factors that mask the phenotype as, for example, the existence of compensatory developmental mechanisms, we expect to get more clear answers testing the memory of transgenic tissue generated by transgenic ES cell lines with the genes for CCK

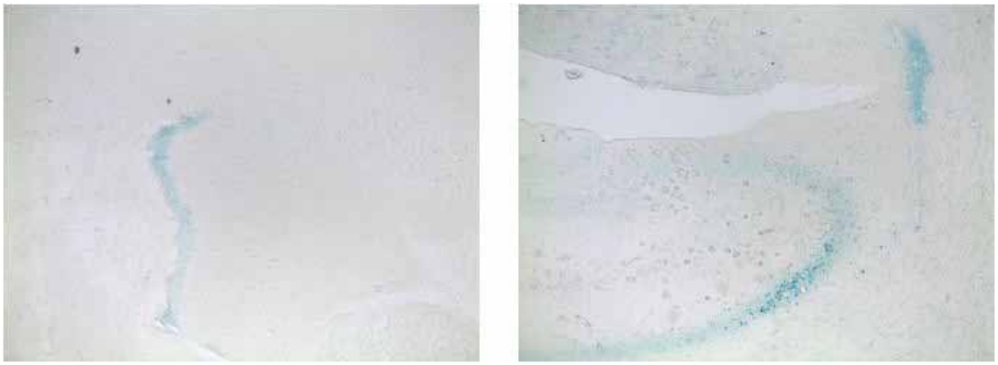
receptors switched-off and developing *in vitro* under stimulation.

At first conditional mutant cell lines were generated. First, we designed targeting constructs for conditional mutagenesis to target both of the genes. We also inserted lacZ expression cassette as a marker to both of the constructs (Figure 5). In general, these replacement vectors (replace endogenous CCK1 or CCK2 receptors) are designed so that in mutant cell lines, instead of endogenous gene, the tauLacZ fusion protein should be expressed. This fusion protein is needed to follow the temporal and spatial activation of these genes and their promoters. Tau signal makes these markers transportable along the axons, and therefore enables visualisation of developing axons. Linearised DNA constructs have been electroporated into ES cells, Neo-resistant clones were isolated and analysed by PCR for the homologous recombination. In positive clones, the targeted locus has been sequenced to verify the recombination.

In addition to the generation of targeting vectors and targeting ES cells, we performed genomic analysis of CCK2 knockout mice. A critical insight revealed was the activation of MAPK kinase pathway (a signal transduction pathway that couples the binding of growth factors to cell surface receptors with intracellular protein activations). The MAPK kinase pathway plays a crucial role in increasing the synthesis of different proteins that are needed for long-term memory. In addition to the generation of the cell lines, we have visualised the gene expression in the brain (Figure 6). We found that CCK1 is expressed in hippocampus.

In conclusion, we have generated conditional transgenic stem cell lines of CCK1, 2 receptors that will be used in the *in vitro* system to generate a synaptic network whose ability to memorise electrical signals will be tested with the differences in the recorded signals between stimulated and non-stimulated networks. We have also detected the expression of these

Figure 6. This figure illustrates the expression of CCK1 Receptor in the mouse brain. In generally, signal was quite weak, but enough to clearly detect CCK1 positive (blue color) neurons in hippocampus. These positive neurons are most likely large pyramidal neurons.



genes in mice and we have found their involvement in signalling pathways related with memory. A similar gene expression analysis in the *in vitro* developing neural tissue will evaluate the *in vitro* system concerning its similarity in reproducing biochemical mechanisms responsible for memory acquisition.

3. Wfs1 knock-out cell line

Wolfram Syndrome (WS) is an autosomal recessive disorder most frequently characterised by Diabetes Insipidis, Diabetes Mellitus, Optic Atrophy and Deafness (DIDMOAD). In addition to these, most of WS patients have highly variable clinical picture with several neurologic abnormalities such as nystagmus, mental retardation, and seisures. Moreover, several studies have shown diffuse and widespread atrophic changes in the brain. In addition to the neurologic manifestations, psychiatric illnesses have often found in WS patients. The most prominent psychiatric manifestations in WS homozygous individuals are depression, violent or assaultive behaviour, and organic brain syndrome. A range of other neurological and psychiatric conditions has also been associated with WS, including short-term memory loss, dementia, psychosis, bipolar disorder, suicide attempts, and impulsive behaviour.

WS is caused by homozygous mutations in WFS1, a gene located at 4p16.1 and

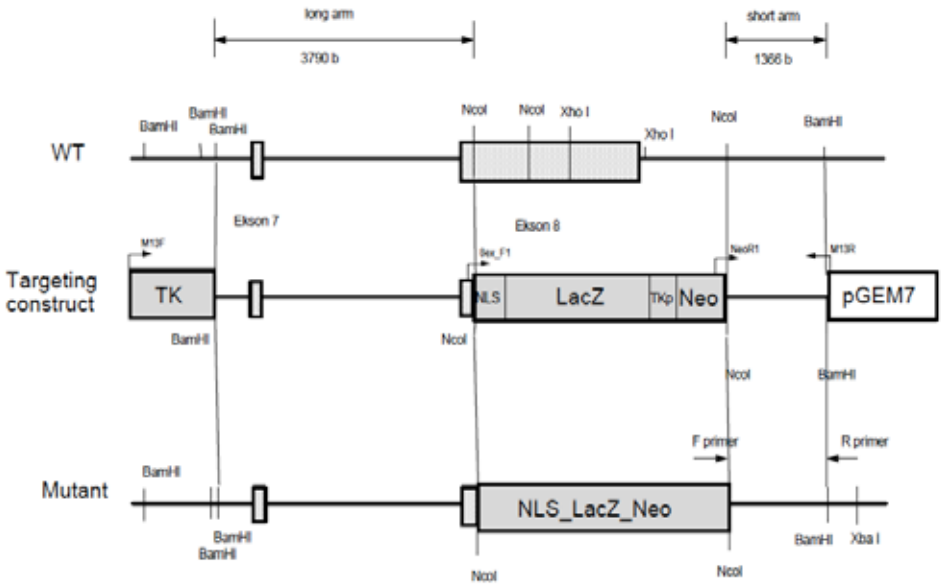


Figure 7. Targeting vector for wfs1 gene targeting and replacement. The lacZ and Neomycin resistance genes replaced 8th exon of the gene. 5' homologous arm (3.8 kb long) consists of the 5' part of the wfs1 gene and ended at the 8th exon NcoI site. 3' homologous arm is the intergenic area after wfs1 gene with the length of 1.3 kb.

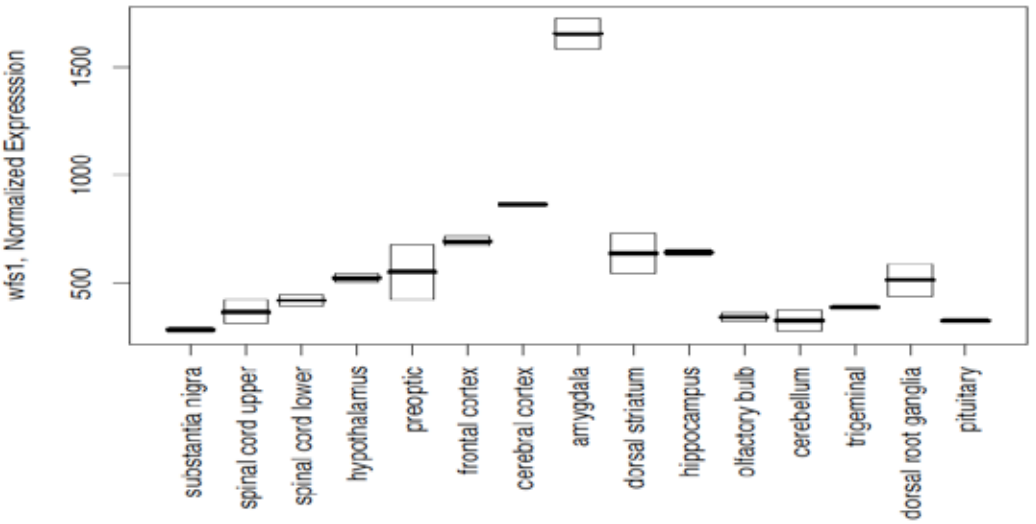


Figure 8. Wfs1 expression is variable in different areas of the mouse brain. We perfomed database analysis based on the Gene Expression omnibus and as a results we found substantial regional differences in the wfs1 expression in the mouse brain. Wfs1 gene is very highly expressed in limbic structures (amygdala, hippocampus) what indicates its role in emotional behavior. Wfs1 is highly expressed also in the cortical regions, and therefore it is involved also in the cognitive functions.

composed of seven coding exons. WFS1 encodes wolframin, an 890 amino acid glycoprotein that localises primarily in the endoplasmic reticulum, where it has been shown to participate in the regulation of cellular calcium homeostasis.

To generate transgenic cell lines, ES cells were cultivated until almost confluent, and then targeting vector (Figure 7) was introduced to the cells with electroporation. Positive clones survived G418 selection, as these had Neo-resistance cassette inserted. 50 positive clones were picked and screened by PCR. Out of 50, 2 clones were preserved and used for blastocyst microinjections. Our targeting vector was designed to replace the 8th exon of wfs1 gene. As the majority (70%) of this protein is coded by 8th exon, this replacement removes most of the protein and invalidates the function of wfs1 gene. Moreover, LacZ gene in the targeting vector forms fusion protein with the 5' part of the wfs1 protein, and therefore its expression is controlled by the natural signals for wfs1 gene. This fusion protein can be used to visualise the endogenous expression of Wfs1 gene. We have also checked the expression of Wfs1 gene in the central nervous system (Figure 8).

The cell lines will be used in the subsequent tasks in the development of transgenic neural tissue in the *in vitro* system, and we will test the electrical activity of

the synaptic network. Since the transgenic mice have shown memory/learning impairment, we expect that differences will be found in the response of the transgenic neural tissue to stimulation relative to that of the normal tissue, as determined in Chapter 3. In addition, similar biochemical tests performed in the *in vitro* developing transgenic neural tissue will be used to evaluate the similarity of the *in vitro* and *in vivo* tissues.

Having determined the differences in response to stimulation signals, we will evaluate the *in vitro* system as an alternative to animal testing. Instead of knocking out genes that are supposed to influence memory/learning and observing their impairment in animals, we could use the knock-out cell lines to generate *in vitro* neural tissue and evaluate from the synaptic network electrical activity whether the knocked-out genes play a role in memory/learning.

C. Neurotoxicity

The purpose of these activities is to bridge the gap between *in vitro* tests and *in vivo* ones that use animals. The *in vitro* tests developed until now that are based at the cell, rather than tissue, level (cells do not have "memory", which is a property of synaptic networks) can provide answers for the effect of neurotoxic compounds in various biochemical mechanisms at the cellular level. However, the extrapolation of results of these tests to the effect of neurotoxic compounds at the level of human behaviour is not possible. For such effects, *in vivo* tests are performed in animals. However, if an *in vitro* system could test the effect of neurotoxic compounds in behavioural-like functions of humans, a step towards the replacement of animals can take place. Due to the complexity of the biochemical mechanisms on which memory relies, several tests with different endpoints are usually performed in cell-based systems (batteries). Are these endpoints, however, complementary and sufficient to predict a behavioural damage? No answer can be given at this moment, because no system exists in which tests of biochemical as well as behavioural parameters could be performed to correlate their relation. The check of the complementarity of the batteries of tests in order for their results to be extrapolated to behavioural effects will be undertaken with the *in vitro* memory tests performed in the *in vitro* system. The memory effects, as seen in the differences of the recorded signals between developing synaptic network in the presence or absence of neurotoxin, will be correlated with biochemical tests.

Neurotoxic compounds with known effect on memory/learning, together with the biochemical mechanisms involved in their effect, have been selected. The compounds included are: alcohol, solvents, beta-amyloid, arsenic, lead, aluminium, manganese, and mercury. We have also selected some compounds such as aluminum, which impairs the long-term-potentiation. This is the phenomenon that we induced in the *in vitro* developing network with stimulation, with the distributed stimulation and with the addition of retinoic as mention in Chapter 3, which proved to

be a successful strategy to detect difference in the recordings between stimulated and non-stimulated networks. We assume, therefore, that if a neurotoxic compound interferes with this phenomenon, there will be an effect in the differences of the signal features between the stimulated and non-stimulated network. This difference is a global one, spread throughout the network, and determines its synaptic connectivity. Such an effect is therefore at a higher level—the level of the network—compared with the lower-level effects at the neuron level. It could be particularly useful to combine low-level biochemical effects with high-level ones, such as the long-term-potentiation, to examine its change with the stimulation, and the appearance of such change with the ability of memory/learning in the presence of neurotoxins that interfere with this basic for memory phenomenon. We have also detected other compounds that interfere with molecules involved in long-term-potentiation, such as NMDA, solvents, and lead. The above-mentioned neurotoxicants interfere with lower-level biochemical/physiological mechanisms such as oxidative stress, neurite outgrowth, apoptosis, changes in protein phosphorylation, etc. This will give us an indication of which biochemical mechanisms could be complementary to lead to memory defects.

Two types of experiments will be performed in the third period of the project, apart from the experiments in the *in vitro* system where signals in the presence of neurotoxins will be recorded and analysed:

a) Biochemical experiments of neurotoxicity.
b) Biochemical experiments specific of general toxicity, to distinguish between cytotoxicity that can be induced by many agents and is not useful if we want to study memory defects at the level of the synaptic network (unless we found that is always present but to a low degree, influencing with the cell death at some location of the network, the synaptic network, e.g., disrupting already developed connections that can be seen from the changes of the recorded signals, e.g., decrease of the bursting activity—addressed by the partners BSL and JRC).

We have concluded that the following experiments will be more informative:

Biochemical experiments of neurotoxicity

1. Neurite outgrowth observation to study the influence of the neurotoxic compounds in the neuronal network.
2. PCK activity assay, since PKC protein interferes in the storage of the long-term memory (LTM).
3. Oxidative stress evaluation, given that a change in the natural balance in the neutralisation and elimination of the reactive oxygen species (ROS) can inhibit the normal the cell
   - Glutathion (GSH) quatification
   - Glutathione perodidase (GPx)
   - Superoxide dismutase (SOD).
4. Expression of mRNA for GABA and NMDA receptors (RT, PCR) neuronal marker.
5. GFAP immunohistochemistry (protein level) and mRNA levels (gene expres-

sion) by RT-PCR (glial marker).

6. Kinetics for the expression of mRNA and immunostaining (protein expression) of neuronal markers (NF-68 and NF-200 and/or Tubulin betaIII).
7. Kinetics for the expression of mRNA and immunostaining (protein expression) for neuronal progenitor cell markers (nestin and Sox 10) to cover the early developmental stage such as proliferation of progenitor cells.
8. Expression of mRNA for synaptophysin (pre-synaptic marker) and PSD-95 (post-synaptic marker) to evaluate synaptogenesis.

Biochemical experiments of general toxicity

1. MTT of Alamar Blue to evaluate the dose-dependent curve to establish non-cytotoxic concentrations that will be used further by specific neurotoxicity tests.
2. Neutral Red Uptake (NRU) assay for evaluation of the chemosensitiveness of the neurons to the neurotoxic compounds.
3. BCA test to predict cytotoxixity or necrotic effects of different substances.
4. Cell morphology observation between the different toxicity experiments in order to compare the cell condition and viability with the control culture.

Conclusion

In conclusion, we could say that we have reached all the milestones of the project related with R&D. Important aspects of the progress of the project include the following:

1. We have developed a new hydrogel with properties sufficient for the attachment and growth of neurons generated by ES cells.
2. We succeeded in developing an *in vitro* system composed on neurons grown and differentiated inside a hydrogel placed in a perfusion bioreactor system connected with electrodes, and all the necessary equipment for signal acquisition and analysis.
3. We have found a stimulation protocol that when applied to the developing neurons in the hydrogel synaptic network, influences their development as it is reflected in the recorded signal.
4. Signals recorded in stimulated networks have been statistically analysed in terms of general and synchronised activity at the level of single microelectrodes and microelectrode array, and compared with those of non-stimulated networks.
5. Important differences in the recorded signals have been found and they will be used in the subsequent tasks of applications, transgenics, and neurotoxicity.
6. The preliminary work for the *in vitro* system to be used has been finished, new transgenic cell lines have been generated, and the experiments of neurotoxicity have been designed.

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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: http://www.esnats.eu

Background

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 (R&D) 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 (ES) cells, including:

- Their characteristic property to self-renew, constituting a potentially unlimited source of cells.
- Their pluripotency (i.e., their potential to give to 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.
- At least for murine ES cells (mES cells), their easy genetic manipulation, allowing

use of reporter gene expression as a powerful toxicity testing tool.

Objectives

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 ES cells subjected to standardised culture and differentiation protocols. By using ES cells, both the effects of test substances on the development of organotypic cells from ES cells and on the differentiated organotypic cells can be studied.

Assays developed in ESNATS are therefore based on ES cells in several stages of development as well as differentiated cells, including gametes, cardiomyocytes and neural lineages, complemented with systems for hepatic metabolism. State-of-the-art genomics approaches are used to identify predictive toxicoproteomics and toxicogenomics 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.

The results of ESNATS are expected to have an impact on multiple levels:

- On pharmaceutical R&D, by providing new ES cell technologies, which will facilitate screening and early decision-making of candidate drugs, and in the long-term might contribute to a more rational and effective drug development process.
- On public health, by contributing to the production of safer drugs at lower cost, which will be available much quicker.
- On European stem cell research, by providing new technologies for standardised stable human ES cell culture, improved protocols for human ES cell differentiation, and a unique, world-leading toxicogenomic database.

Furthermore, the new testing rules un-

der the EU regulation on the Registration, Evaluation and Authorisation of Chemicals (REACH) require extensive toxicological safety testing of both existing and new chemical substances, which can also include drug intermediates. The ESNATS project provides valuable information for risk identification in regulatory toxicology. Alternative testing strategies are highly needed in this field of work to limit the number of animal tests required to comply with the REACH regulation.

Experimental Design

The work to be performed in ESNATS is divided into four main research areas, each one representing a sub-project (SP). These SPs are complemented by central work packages (cWPs), which cover transversal scientific aspects of the project (see Figure 1). In addition, the project plan foresees cWPs for the management of the consortium, as well as for training and dissemination work.

On the basis of ES cells and differentiation of ES cells, the following tests and readout systems are under development:

- Preimplantation embryotoxicity test based on undifferentiated hES cells.
- Preimplantation embryotoxicity test based on a hES cell-derived trophoblast model.
- Toxicological testing in an *in vitro* gametogenesis system (based on mES cells).
- Humanised tests for assessing aspects of developmental toxicity *in vitro* for drug safety screening using neural and

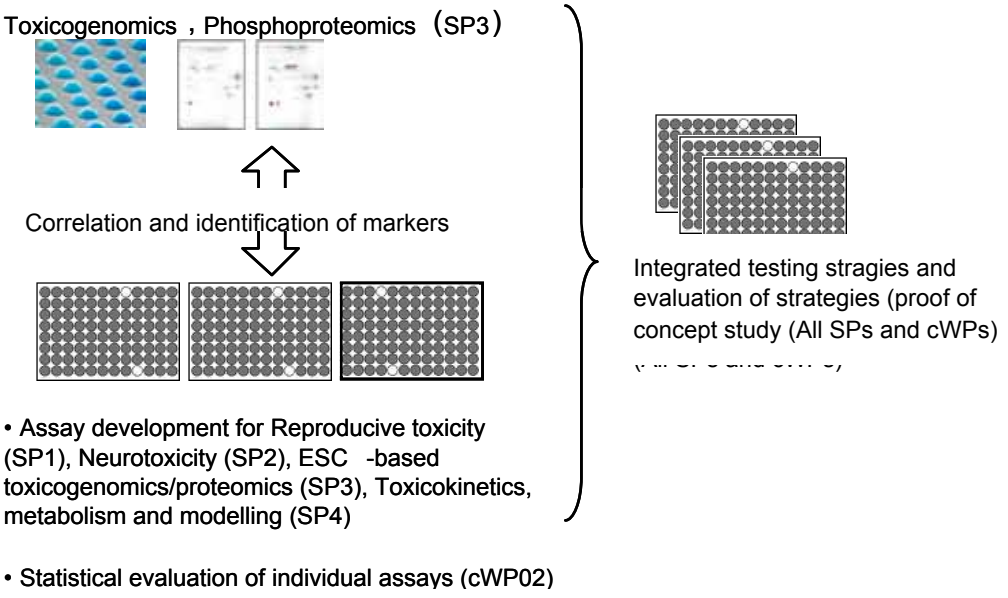

Figure 1. ESNATS main research areas.

cardiac differentiation assays based on hES cells.
- hES cell-based predictive epigenetic profiling and pluripotency reporter screening systems.
- hES cell-based early human developmental toxicity test for increased-throughput testing in early drug discovery.
- Test for developmental neurotoxicity.
- Tests for acute neurotoxicity.
- Functional toxicity in tests integrated neural tissues.
- Developmental toxicogenomics signatures.
- Metabolising *in vitro* systems are for integration into these tests.

The overall test strategy will rely on selected, complementary *in vitro* assays addressing selected toxicological questions (see also "Next Steps").

The ESNATS project has now completed its first two years. Work over this period focused mainly on the preparation of the cell systems as the basis for the test development, which will be fine-tuned to obtain readily usable test systems in year 3. Years 4 and 5 will be mainly dedicated to the combination of the test systems in an overall testing strategy, and the proof-of-concept study, including through blind tests.

More specifically, in the first project year:

- The ESNATS partners initiated the set-up of the *in vitro* assays for reproductive toxicity and neurotoxicity, focusing on the establishment of the underlying ES cell systems, including work on ES cell maintenance and differentiation into the developed target cells.
- Standard operating procedures (SOPs) were developed for several cell systems to standardise differentiation of hES cells into target cells.
- Standard methods for RNA sample generation and processing were developed in order to minimise variability due to sample handling, and quality-control protocols for hES cell cultures were developed to ensure reliability/reproducibility. A database for online tracking of samples and associated data was developed.
- Culture conditions of primary mouse and human hepatocytes were optimised in order to be able to integrate them as metabolising systems into the testing strategies with ES cell-derived cells. Work started on physiologically-based pharmacokinetic (PBPK) modelling.
- A list of test substances was drafted by the SPs, and a first recommendation of selected test substances was made by the Steering Committee, an advisory panel composed of representatives from industry and regulatory bodies. The Steering Committee also provided industrial and regulatory specifications for assay development.
- The user requirements specification for equipment to automate standard protocols was drafted, and passaging methods were identified and evaluated for their applicability to automation.
- Training courses were carried out by the ESNATS partners on phosphoproteomics, microarray expression analy-

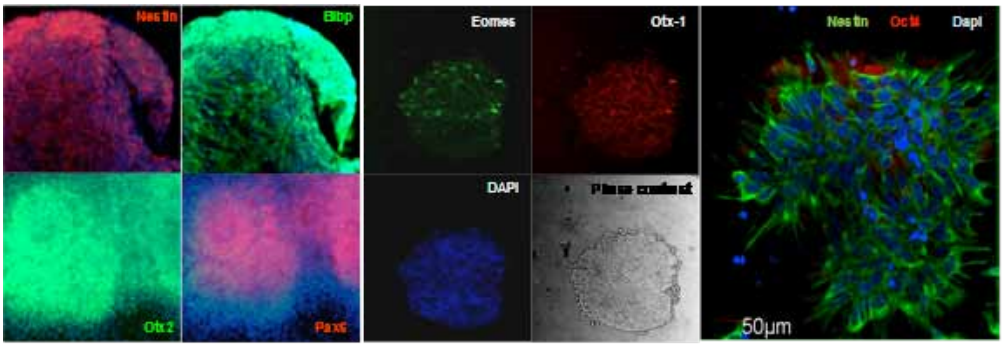

Figure 2. Some of the cellular model systems developed in ESNATS. Left: Expression of markers in neural rosettes. Nuclei are stained blue (Hoechst dye). Source: G. Lazzari. Middle: Marker expression in the standardised trophoblast model system. Source: P. DeSousa. Right: Marker expression in HES1-derived neural progenitors. Source O. Wiser.

sis, and ethical aspects, in order to share a common understanding of issues at stake. A workshop on statistics was organised together with the PREDICT-IV project.
- The ESNATS public website was set up, and the ESNATS flyer was edited and distributed at the first public event, organised by the University of Newcastle upon Tyne in April 2009.

In the second project year, the ESNATS partners maintained their focus on setting up the *in vitro* toxicology test assays.

- ES cell culture and differentiation systems were further developed for use in reproductive, developmental, and neural toxicity testing (see Figure 2). In particular, the differentiation of stem cells into a wide range of cells and tissues was optimised, including neural cell types and engineered neural tissues, for cardiomyogenesis and for (mouse) ES cell-derived spermatogenesis. SOPs for additional cell systems were developed, including for two- and three-dimensional neural tissues.
- The ESNATS proteomics and genomics subproject (SP3) has been analysing sample material prepared by partner laboratories to characterise cell models and identify toxicological signatures. All samples are documented in the online database for sample and associated data management, and then processed and analysed by the toxicogenomics and -proteomics facilities. Proteomics and genomics signatures for selected test substances in individual assays were identified.
- *In vitro* assays will be systematically challenged with extended sets of test substances to enable definition of comparative toxicity signatures. To host the resulting data and permit analysis according to user-defined criteria, a web-based and password-protected tool for genomics analysis was developed and made available to all partners. Simi-

larly, a database for the collection of dose-response data and online tools for their analysis was developed and made available.

- In the area of metabolising systems, de-differentiation and loss of metabolic activity—one of the major hurdles of using primary hepatocytes as a metabolising system—has been investigated and the most critical mechanisms responsible for dedifferentiation were identified, enabling approaches to better maintain the differentiated state. Stem cell-derived metabolically-active cells have been generated, but major differences remain as compared to primary hepatocytes and further improvements are necessary.
- An integrated approach of *in vitro* testing and PBPK modelling to be used to predict *in vivo* effect levels for reproduction toxicity and neurotoxicity has also been developed.
- The list of test substances for test development has been extended and finalised based on the input of the SPs, which was reviewed by the Steering Committee and a recommendation of selected test substances was provided.
- In the area of automation and scale-up, enzymatic dissociation systems were evaluated as a prerequisite to scaling up of hES cell culture. Based on the user requirements specification, a concept for a medium-scale robotic system to automate standard ES cell culture has been developed. Regarding banking, a comparative study on feeder-based and feeder-free systems is currently being completed.

Results

Work has for the most part progressed as expected. Protocols for the ES cell-based cellular model systems were successfully developed and challenged with sets of test substances. All major deliverables planned for the first two project years as building stones for subsequent work have been achieved:

- Initial SOP for Engineered Neural Tissue.
- Report on the characterisation of hES cell-derived neural cells and the most suitable predictive endpoints for neuronal teratogenicity.
- Protocols for the spontaneous multilineage differentiation of hES cells in 96-well plates as an *in vitro* model for early human development suitable for M/HTS.
- SOP for murine mixed cultures.
- *In vitro* screening tests using drug candidates in the gametogenesis system.
- Definition of hES cell trophoblast model structure.
- SOP for human neural precursor cells.
- SOP for human mixed cultures.

Next Steps

In the coming third year, cell systems will be fine-tuned and adjusted to become readily usable test systems to be challenged with full sets of reference substances. A specific task force has been set up in the project, which aims to define the overall test strategy and to identify the test systems

on which the project will focus. The overall integrated testing strategy to be applied in the two last years in the project (years 4 and 5) will be based on the selection of the most suitable *in vitro* test systems (considering criteria such as reliability and robustness of the protocol), identification of targets to be predicted by the assays, definition of the appropriate readouts, as well as the combination of these, such as toxicogenomics, proteomics and functional readouts. In particular, microarrays will be used to identify expression signatures characteristic of toxicological responses that will be used as the basis for readout techniques such as quantitative PCR.

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2.2 Integrated testing strategies

ACuteTox

Optimisation and pre-validation of an *in vitro* testing strategy for predicting acute toxicity

Contract number:	LSHB-CT-2004-512051
Project type:	Integrated Project (FP6)
EC contribution:	€ 9 000 000
Starting date:	1 January 2006
Duration:	60 months

Website: http://www.acutetox.eu

Background

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) and United Nations' Globally Harmonised System (GHS) hazard classes. The extensive amount of work performed since the 1970s has led to the large number of existing *in vitro* models for acute toxicity testing. Many studies have shown good correlation between *in vitro* basal cytotoxicity data and rodent LD_{50} values. However, a certain number of misclassifications will occur when the existing tests are used. ACuteTox aims to identify factors that can optimise the *in vitro-in vivo* correlation for acute oral systemic toxicity.

Objectives

The main objectives of the project include the compilation, evaluation and/or generation of high-quality *in vitro* and *in vivo* data 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). Moreover, innovative tools (e.g., cytomics) and new cellular systems for anticipating animal and human toxicity are explored. Ultimately, the goal is to design a simple, robust and reliable *in vitro* test strategy amendable for robotic testing, associated with the prediction models for acute oral toxicity.

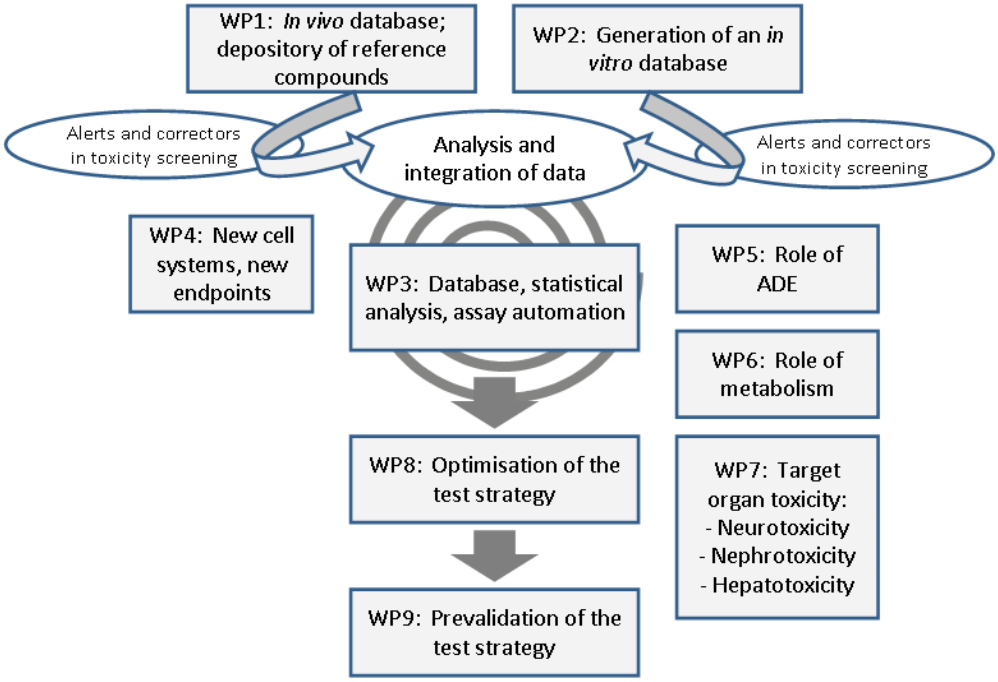


Figure 1. The general structure of the ACuteTox project.

Experimental Design

In the first part of the project, 97 reference chemicals were selected and tested using 6 basal cytotoxicity assays. Moreover, human and animal *in vivo* data for these substances were collected from the scientific literature. This allowed to identify the outliers from the *in vitro*/*in vivo* correlations, both using calculated human lethal concentrations (LC_{50}) and rat oral LD_{50} data. These outliers, as well as a balanced number of non-outliers from the reference set, were then tested in several *in vitro* and *in silico* models. By the end of 2007, 57 reference chemicals were tested in a number of functional tests covering absorption, distribution, excretion, metabolism, and specific organ- and system-toxicity, such as haemato-, neuro-, nephro-, and hepatotoxicity. The overview of project activities and interaction between the different work packages is presented in Figure 1.

The data generated were stored in a novel internet-based database (AcutoxBase) developed within the project, and were used to assess the within-laboratory variability, the preliminary predictive capacity, and in some cases also the between-laboratory variability of each *in vitro* assay.

A Partial Least Square Analysis (PLS) was first performed on all data generated, the *in vitro* IC_{50} values calculated in each laboratory were correlated with human LC_{50} values and rat oral LD_{50} values, and the best combinations of *in vitro* tests that

gave a relatively good correlation with *in vivo* (rat and human) data were identified. A more extensive analysis was performed to identify possible strategies that would allow classification of chemicals into the official acute oral toxicity categories (EU and GHS). For this purpose the Classification and Regression Trees (CART) was used as the classification algorithm of choice.

Finally, 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 *ad hoc* 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 *in vitro* assays evaluated in the ACuteTox project are presented in Table 1, which presents the status of the assays, indication whether within laboratory reproducibility, between laboratory reproducibility and preliminary predictive capacity were evaluated, and if the assay was selected as candidate for the tiered testing strategy.

Results

As a result of the above statistical analysis, potential assays for the ACuteTox testing strategy were selected according to their reproducibility and reliability as well as to their potential to classify chemicals into the official acute oral toxicity classes (GHS and CLP). The best-performing *in vitro* assays, which were selected as candidates for the final tiered testing strategy, are as follows:

1. The Neutral Red Uptake assay using the 3T3 fibroblast cell line (3T3/NRU) (Figure 2).
2. The Cytokine Release Assay using human whole blood (IL-1, IL-6, TNF-

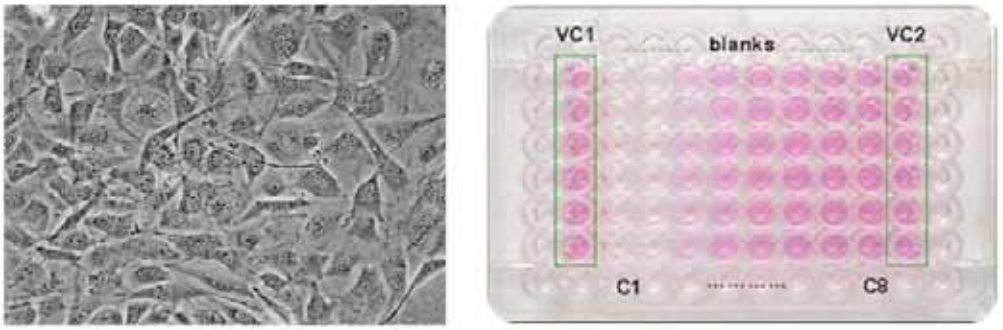


Figure 2. The Neutral Red Uptake assay using the 3T3 mouse fibroblast cell line. The cells are incubated in 96-well plates with increasing concentrations of the tested compound and viability is measured after 48 hours using the Neutral Red Uptake assay.

alpha).

3. Inhibition of colony forming unit efficiency in human cord blood-derived cells stimulated with CFU-GM (CBC/CFU-GM) (Figure 3).
4. Gene expression (GFAP, HSP-32, MBP and NF-H) in primary rat brain aggregate cultures (Figure 4).
5. Uridine incorporation measuring the total mRNA synthesis in primary rat brain aggregate cultures (Figure 4).
6. 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.
7. Cytomic panel for cytotoxicity screening (intracellular Ca^{2+} levels, mitochondrial membrane potential, plasma membrane potential) in HepG2, SH-SY5Y and A.704 cells.
8. The MTT assay using primary rat hepatocytes (Figure 5).
9. Kinetic parameters: volume of distribution, protein binding, clearance, and oral absorption (Caco-2 cells) for the estimation of the oral dose from the effective concentration observed *in vitro*.

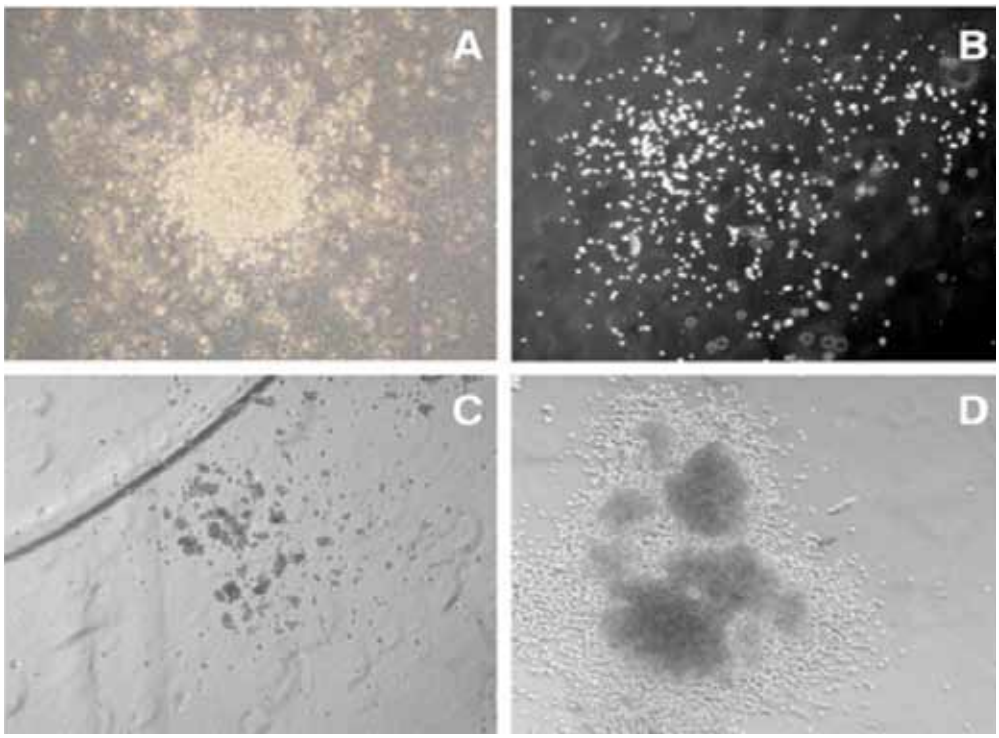


Figure 3. Microphotographs of different types of CFU-GM colonies. (A) Compact colony: with a central dense nucleus and a peripheral halo. (B) Diffuse and spread colony without apparent nucleus. (C) Burst-forming-like colony: multifocal colony with several aggregates, with of without a peripheral halo. (D) Multicentric colony with two or more dense nuclei (Cerrato et al., 2009).

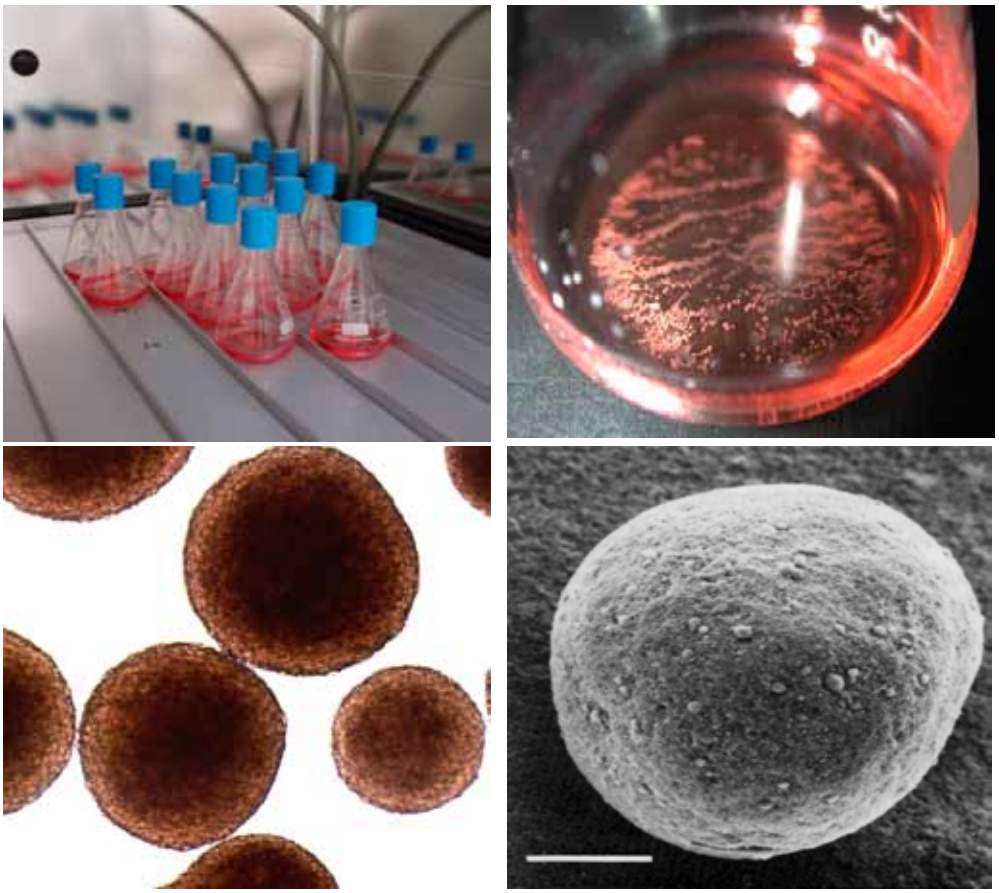
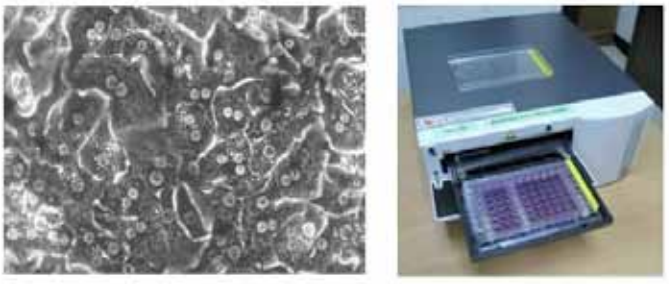


Figure 4. Re-aggregated brain cell cultures. Top row: the cultures are kept in Erlenmayer flasks under constant rotation in an incubator. Bottom row: Microphotographs of rat brain aggregates (20 days *in vitro*).

Figure 5. The MTT assay performed on cultures of primary rat hepatocytes. The cells are incubated in 96-well plates with increasing concentrations of the tested compound and cell viability is measured after 24 hours using the MTT assay, based on the ability of a mitochondrial dehydrogenase enzyme present in viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals, which are solubilised in DMSO. The amount of formazan formed is measured colorimetrically in a multi-well scanning spectrophotometer.



10. The estimation of compound passage through the blood-brain barrier using neuronal networks (for neurotoxicity assays) (Figure 6).

These selected tests are now challenged with a new set of 32 coded chemicals in a prevalidation study.

It has to be noted that the time left for prevalidation was shorter than initially planned. Thus, the transfer of methods to a second independent laboratory is not feasible, as this requires appropriate training of the laboratories involved. For the same reasons, for most the methods the assessment of the between-laboratory reproducibility will not be performed.

Next Steps

These selected tests are now challenged with a new set of 32 coded chemicals in a prevalidation study. During this last phase of the ACuteTox project, the main focus will be on the assessment of the predictive capacity of the proposed tiered testing strategies and the identification of the combination that gives the best prediction.

The expected result of the project is a prevalidated tiered-testing strategy containing up to 10 different *in vitro*/*in silico* assays that can be used by industry, and in particular in the chemical and pesticides sector, as a (partial) replacement of the *in vivo* acute oral toxicity test. However, before the strategy will be proposed for regulatory use, it will have to be formally validated (completion of all modules of the ECVAM's modular approach). Therefore, a formal submission to ECVAM of a proposal for complete validation of the testing strategy is foreseen at the end of the project.

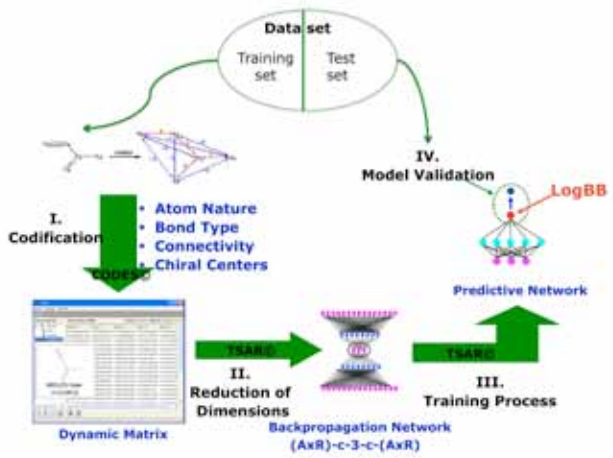


Figure 6. Strategy used to develop a neural network model to predict the passage of the blood-brain barrier. The key steps of this methodology are: I) codification using CODES programme, II) reduction of dimensions of the dynamic matrix, III) training of the neural model and IV) model validation.

Table 1. In vitro assays evaluated in the different Work Packages (WPs) of the ACuteTox project. The table presents the status of the assays, indication whether within-laboratory reproducibility, between-laboratory reproducibility, and preliminary predictive capacity were evaluated, and if the assay was selected as candidate for the tiered testing strategy.

In vitro assay	Test system	Within-lab reproducibility	Between-lab reproducibility	Preliminary predictive capacity	Candidates for tiered testing strategy
Cytotoxicity					
ATP content	HL-60 human cell line	X			no
Protein content (CBQCA assay)	HepG2 human hepatoma cell line	X			no
Protein content (CBQCA assay)	Fa32 rat hepatoma cell line	X			no
Neutral red uptake	Balb/3T3 mouse fibroblasts	X	X	X	**YES**
Neutral red uptake	Normal human keratinocytes	X	X	X	no
Neutral red uptake	Fa32 rat hepatoma cell line	X			no
New cell systems, new endpoints					
Multiplexed flow cytometry analysis of cytokine release (IL-12p70, IFN-γ, IL-2, IL-10, IL-8, IL-6, IL-4, IL-5, IL-1β, TNF-α, TNF-β)	Human peripheral blood mononuclear cells (PBMC)	X		X	**YES**
ELISA analysis of cytokines IL-5, IFN-γ and TNF-a release	Human peripheral blood mononuclear cells (PBMC)	X		X	no

In vitro assay	Test system	Within-lab reproducibility	Between-lab reproducibility	Preliminary predictive capacity	Candidates for tiered testing strategy
Cytokine release (IL-1, IL-6, TNF-a)	Human whole blood			X	**YES**
Colony forming unit-granulocyte/ macrophage (CFU-GM)	Human cord blood cells	X	X	X	**YES**
Cytomic Panel for Cytotoxicity Screening including: · intracellular Ca^{2+} (Fluo-4 probe) · mitochondrial membrane potential (rhodamine123) · plasma membrane potential (DIBAC probe) · intracellular lipid content (BODIPY probe)	Performed in three cell lines: · A.704 kidney adenocarcinoma · HepG2 human hepatoma cell line · SH-SY5Y human neuroblastoma cell line	X		X	**YES**
Cytomic Panel for Oxidative Stress Screening including: - intracellular peroxides - mitochondrial generation of superoxide - intracellular levels of the oxidized DNA base 8-Oxo-Guanine	Performed in three cell lines: · A.704 kidney adenocarcinoma · HepG2 human hepatoma cell line · SH-SY5Y human neuroblastoma cell line	X		X	**YES**

In vitro assay	Test system	Within-lab reproduci-bility	Between-lab reproduci-bility	Preliminary predictive capacity	Candidates for tiered testing strategy
Biokinetics					
PAMPA assay	Not available				no
Intestinal absorption (permeability assay)	Caco-2 intestinal cell line*	X	X	X	**YES, a)**
Intestinal absorption (toxicity assay - ^{14}C-Mannitol)	Caco-2 intestinal cell line*	X	X	X	no
Intestinal absorption (toxicity assay – Lucifer yellow)	Caco-2 intestinal cell line*	X	X	X	no
Blood-brain barrier passage (permeability assay)	Blood-brain barrier model **	X			no
Blood-brain barrier (toxicity assay – Lucifer yellow)	Blood-brain barrier model **	X			no
Blood-brain barrier (toxicity assay - ^{14}C-sucrose)	Blood-brain barrier model **	X			no
Aqueous solubility	Not available	See note a)			**YES**
Plasma protein binding	Pooled human plasma***	See note a)			**YES**
Chromatographic Hydrophobicity Index (CHI)	Not available				
Metabolic stability	Rat liver microsomes****	See note a)			**YES**
Metabolic stability	Human liver microsomes****	See note a)			**YES**
Metabolic stability	Primary rat hepatocytes	See note a)			**YES**

In vitro assay	Test system	Within-lab reproduci-bility	Between-lab reproduci-bility	Preliminary predictive capacity	Candidates for tiered testing strategy
Metabolic stability	Cryopreserved human hepatocytes	See note a)			**YES**
Neurotoxicity					
Alamar Blue assay	Primary rat cerebellum granule cells (CGCs)	See note b)			no
Mitochondrial membrane potential	Primary rat cerebellum granule cells (CGCs)	See note b)			no
AChE inhibition	Pure enzyme	See note b)			no
AChE inhibition	SH-SY5Y human neuroblastoma cell line	X			no
LDH leakage	SH-SY5Y human neuroblastoma cell line	See note b)			no
LDH leakage	Rat brain slices	See note b)			no
Ca^{2+} overload	SH-SY5Y human neuroblastoma cell line	See note b)			no
GABA-A receptor function	Primary mouse cortical neurons	X		X	no
GABA uptake	Primary mouse cortical neurons	See note b)			no
Cell membrane potential (CMP)	Primary mouse cortical neurons	See note b)			no
Glutamate uptake	Primary mouse cerebellum granule cells (CGCs)	See note b)			no
Cell membrane potential (CMP)	SH-SY5Y human neuroblastoma cell line	X		X	**no**

In vitro assay	Test system	Within-lab reproducibility	Between-lab reproducibility	Preliminary predictive capacity	Candidates for tiered testing strategy
Noradrenalin uptake	SH-SY5Y human neuroblastoma cell line	See note b)			**no**
Voltage operated Ca^{2+} channel function	SH-SY5Y human neuroblastoma cell line	See note b)			**no**
Acetylcholine receptor function	SH-SY5Y human neuroblastoma cell line	See note b)			**no**
Gene expression (GFAP, MBP, NF-H, NF-M, PPAR-gamma, HSP-32, iNOS)	Rat re-aggregated brain cells culture	X		X	**YES**
Enzyme inhibition/ activation (ChAT, GS, AChE, LDH, 2,3-CNP)	Rat re-aggregated brain cells culture	See note b)			no
Methionine uptake (protein synthesis)	Rat re-aggregated brain cells culture	X			no
Uridine uptake (RNA synthesis)	Rat re-aggregated brain cells culture	X		X	**YES**
2-deoxyglucose uptake	Rat re-aggregated brain cells culture	X		X	no
Caspase-3 mRNA expression	Primary rat cerebellum granule cells (CGCs)	X			no
MTT assay	Primary rat cerebellum granule cells (CGCs)	See note b)			no

In vitro assay	Test system	Within-lab reproducibility	Between-lab reproducibility	Preliminary predictive capacity	Candidates for tiered testing strategy
Glutamate induced cytosolic Ca^{2+} increase	Primary rat cerebellum granule cells (CGCs)	See note b)			no
ROS production	Primary rat cerebellum granule cells (CGCs)	See note b)			no
LDH release	Primary rat cerebellum granule cells (CGCs)	See note b)			no
Microarray quantitative mRNA expression analyses of 31 genes	Primary rat cerebellum granule cells (CGCs)	See note b)			no
Nephrotoxicity					
Alamar Blue	LLCPK-1 renal epithelial *cell* line	X		X	no
Transepithelial electrical resistance (TEER)	LLCPK-1 renal epithelial *cell* line	X		X	no
Hepatotoxicity, metabolism and bioactivation					
MTT assay	Rat hepatocytes	X	X	X	**YES**
MTT assay	Balb/3T3 mouse fibroblasts	X	X	X	no
MTT assay		X	X	X	no

[a] These assays are providing input data for the PBBK modelling (biokinetics).
[b] Only 23 chemicals have been tested.

** Three different protocols for culturing Caco-2 cells and for the assessment of toxicity and prediction of intestinal absorption in Caco-2 cells have been evaluated in WP5. The cell models were: Caco-2/TC-7 clone, CacoReady™ (Advancell, Spain), Caco-2 from ATCC.*
*** Two different models of blood-brain barrier (BBB), as well as three different protocols for the assessment of toxicity and prediction of BBB passage, have been evaluated in WP5.*
**** Two different protocols for plasma protein binding were used in WP5.*
***** Two different protocols for rat and human microsomal stability were used in WP5.*

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2.3 ‘omics, bioinformatics & computational biology

Sens-it-iv

Novel testing strategies for *in vitro* assessment of allergens

Contract number:	LSHB-CT-2005-018681
Project type:	Integrated Project (FP6)
EC contribution:	€ 10 999 700
Starting date:	1 October 2005
Duration:	66 months

Website: http://www.sens-it-iv.eu

Background

With some forms of allergy increasing, massive resources are invested worldwide to investigate which compounds are the culprits and why otherwise harmless compounds elicit adverse immune responses.

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 regulation "REACH" is expected to consume millions of animals per year. Conversely, several EU directives call for significant reductions or even a complete ban on animal testing (e.g., for cosmetic products and ingredients since 2004 and 2009, respectively).

Therefore, 26 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 the allergenic potential of compounds. This is seen in relation with the use of safe ingredients by the chemical, cosmetic, and pharmaceutical industries.

Objectives

The overall goal of Sens-it-iv is to develop novel *in vitro* tools that can be incorporated into testing strategies to replace animal experimentation for identifying skin and respiratory sensitisers (chemicals as well as proteins), and the potential of testing of the

sensitising potency of existing and new chemical entities produced by the European industries, for classification and labelling as required by the new EU legislation on chemicals, and for the purpose of risk assessment as required by the 7th Amendment to the Cosmetic Directive.

The ultimate deliverables (month 66) of the project are *in vitro* tests that are ready for prevalidation by the European Centre for the Validation of Alternative Methods (ECVAM). Prevalidation as such is outside the scope of Sens-it-iv. "Ready for prevalidation" implies that the tests have to be relevant and meaningful, based upon key mechanisms-of-action and address specific questions. Standard operation procedures (SOPs) with detailed performance criteria have to be established.

A. Scientific Objectives (Science Module)

Sens-it-iv aims to acquire a solid understanding of the processes (and the underlying mechanisms) occurring *in vivo* when tissue is challenged by a potential sensitiser. This understanding is to be used for the development of cell-based tests with *in vivo*-functionality. The *in vivo*-functionality will assure the relevance and meaningfulness of the *in vitro* test systems.

To this end, Sens-it-iv will focus on the following measurables:

- The *ex vivo* phenotypic characteristics of human epithelial cells (EC), antigen presenting dendritic cells (DC), and effector T-cells, addressed using functional genomics, proteomics, and immunohistology before and after chemical challenge of tissue slices.
- *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.
- Description of the chemical features related to 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 will be characterised. Sens-it-iv will address the processes of bio-activation and hapten formation using advanced metabolomic and proteomic technology.

The acquired knowledge is required to identify (i) available cell sources with relevant *in vivo*-like characteristics, (ii) cell culture conditions supporting cellular phenotypic stability and sustainability, and (iii) cellular and/or excreted markers specifically relevant for sensitisation.

B. Technological Objectives

Sens-it-iv aims to develop assay systems that model sensitisation, rather than irritation and toxicity of chemicals and proteins. To meet this objective, the following measurables will be persued:

- *An inductive database on the acquired scientific data, including all available literature information.* This database should facilitate identification of mechanisms and biological markers specifically involved in skin and respiratory sensiti-

sation by allowing queries for data patterns and predictive models.

- *Cell-based predictive assays* (prototypes) developed by implementation of the cellular and excreted markers suggested by bioinformatics to represent key mechanisms of sensitisation. The impact of selected compounds on these assays will be described using genomics and other relevant experimental strategies. Cell culture conditions will be adjusted to allow *in vivo*-like cellular phenotypes.
- *A platform for the development of* in vitro *cell-based test systems/strategies*, by a process of refinement and optimisation of the assays (measurable 2) using a selection of well-defined chemicals and proteins. The systems will be ready for prevalidation and ultimate approval by ECVAM. The strategies will include *in vitro* test systems assessing bio-activation and hapten-formation.

Experimental Design

The Sens-it-iv Sphere

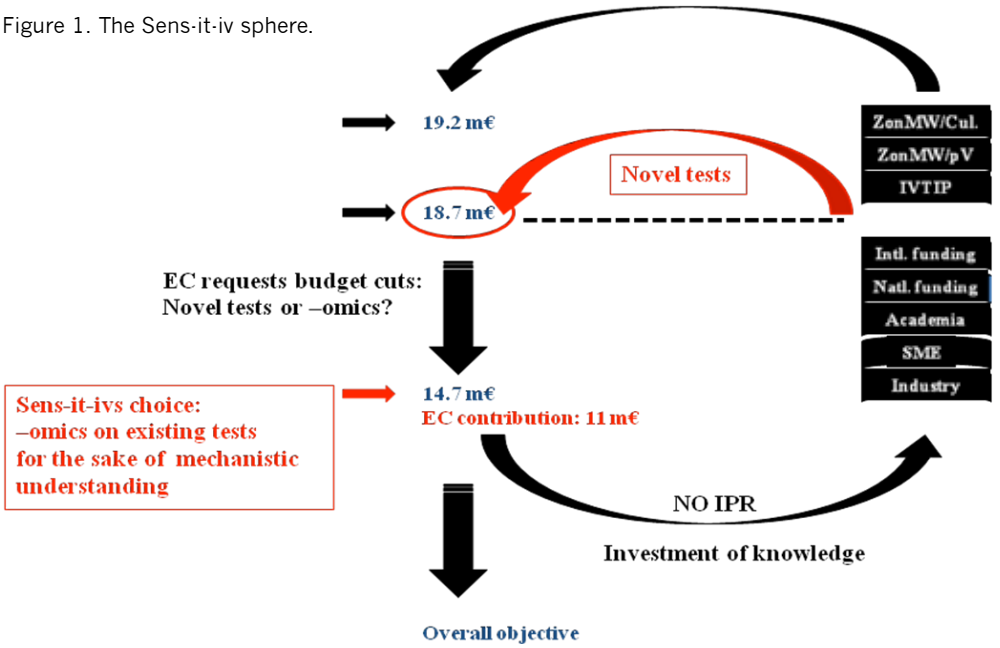
The project that originally was submitted to the European Commission (EC) was budgeted for 18.7 million €. The request by the EC to reduce this budget to 14.7 million € forced the Consortium to chose between "developing novel tests but using existing markers" and "subjecting existing tests to extensive '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, which 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 in terms of 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 (0.5 million €) was assigned by the Dutch funding agency ZonMW to run a prevalidation study on a selected test approach, and to transfer a 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 (through 31 March 2012).

In order to make it possible to manage such a large and complex project properly, the Sens-it-iv project was split in 3 mod-

Figure 1. The Sens-it-iv sphere.



ules, 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 (GA) in October 2007 and 2008 (Figure 2).

1. *Training for prevalidation (Months 24-38):* This training was introduced in order to familiarise the partners with the procedures of prevalidation before tests were selected for evaluation and refinement (Technology Module). Three very simple dendritic cell (DC)-based assays were selected. The result of this bridging activity was an understanding for the requirements of prevalidation, 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 prevalidation project funded by ZonMW.
2. *6-month extension in project duration (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 Sens-it-iv project. The outcome of these discussions will be published in a peer-reviewed journal.
3. *Extension of specific R&D activities:* In general, basic research was to be performed during the first three years only (2005-2008). However, specific activities were allowed to continue (Science Module). These activities were selected because further re-

search could give a better understanding of the underlying mechanisms resulting in better tests.

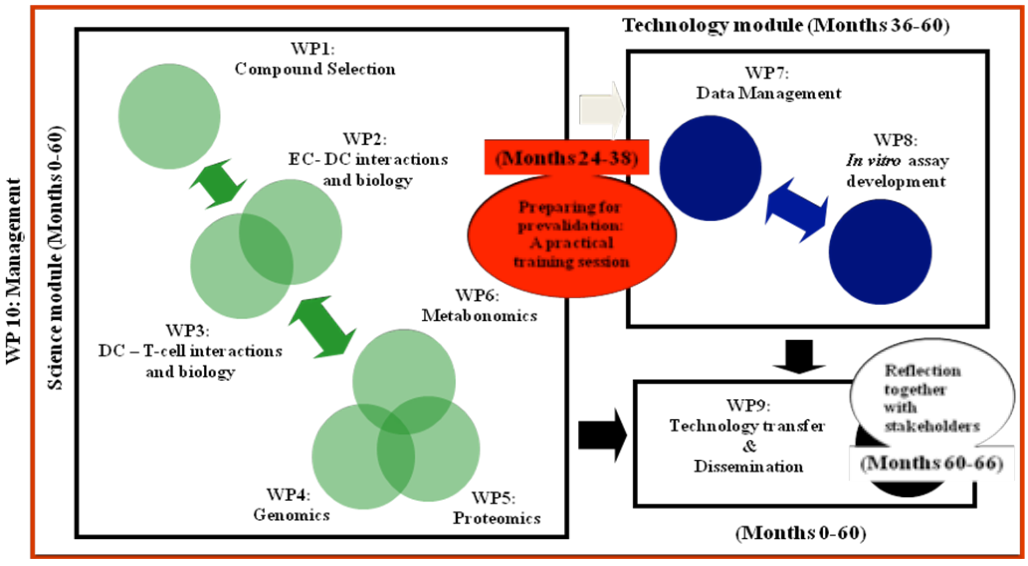
A. Science Module (Month 0-60) – Basic Research

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 (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 pri-

Figure 2. The updated modular structure of the project.



marily 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 coordination 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, (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 activities include:

WP2:

- DC migration (until October 2009, review by GA)
- Interactions between epithelial cells (EC) and DC (until October 2010, review by GA).

WP3:

- Innate immune responses to chemicals (until October 2010, review by GA).

WP6:

- *In vitro* bio-activation (until May 2010, mid-review by GA).

WP4 and WP5:

- marker identification by a coordinated genomics-proteomics (until May 2010, mid-review by GA).

B. Technology Module (Month 36-60)

The aim of the Technology Module was to collect and implement the knowledge acquired by the Science Module.

WP7 has collected since the outset of the project relevant, properly evaluated information from each WP (e.g., SOPs, 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 op-

timise this process, a tight collaboration with the FP7 OpenTox project was established.

WP8 involves 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 prevalidation 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.

C. Communication, Technology Transfer & Dissemination (Month 0-66)

WP9 has assured visibility of Sens-it-iv by means of a proactive strategy. The ultimate goal is still to ensure early awareness and easier adoption of the *in vitro* test methods by various industry sectors as key end-users, together with academia and regulators. A website (*Sens-it-iv.eu*; 6499 unique visitors in 2009) was established and maintained. This website consists of a public site for dissemination to the outside, 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). COLIPA, ecopa, ECVAM and IVTIP (all Sens-it-iv partners) 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 respective 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 assess opportunities (e.g., e-learning) to assure that the knowledge and expertise collected and acquired by the project persists after March 2010 (The Sens-it-iv Legacy).

D. Project Management (Month 0-66) as Relevant for the Experimental Design

Project management was taken care of by the Coordinator, assisted by the Vice-coordinator, 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 dissemi-

nation 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 year-and-a-half 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; WPs 2, 4 and 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 two 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.

With respect to compound selection, the contribution of WP1 was finalised with the release of the extended list of compounds, now comprising 50 chemicals and 21 proteins for extensive evaluation of the selected tests (Table 1). This is an extension with 20 chemicals and 16 proteins since the 2009 GA. Moreover, guidelines for dissolving chemicals were produced specifically for each assay investigated. These guidelines are available on the internal web site (WP7).

Results

A. Final List of Compounds

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)

Respiratory	Skin	Controls
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-phenylenediamine (NPD)	Tween 20 (T20)
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 *		

* Industrial enzymes with various sensitising potency; origin and batch numbers will be revealed by Novozymes when appropriate. All enzymes are supported by epidemiological follow-up in humans (production plants) and/or animal studies (guinea pig GPMT, mice intranasal (MINT) and an in-house modified LLNA).

B. Novelty

WP1 has also expanded the database with information about chemicals and proteins, and test systems with relevance for sensitisation. This information was used to help Sens-it-iv focus on novelty in terms of tests systems and markers, by identifying activities that were overlapping with research and development ongoing outside this project without having significant added value. Tests that were down-prioritised for further development by the GA (October 2009) are listed in Table 2.

C. Tests

Test 1: Identification of skin sensitisers using the cell line NCTC2544 with IL-18 expression as read-out

It was previously established that human keratinocytes (hKC) *in vivo* and *in vitro* respond to sensitisers by increased production of IL-18. This mechanism-of-action was also observed when the hKC cell line NCTC2544 was exposed to chemical sensitisers. In addition, this cell line was described to express the CYP activity, which required for *in vitro* bio-activation of pro-haptens. Whether or not this is the only human cell line with these *in vivo*-like functionalities was not assessed.

Previously, data obtained with 3 skin sensitisers (CIN, DNCB, and TMTD), 3 lung sensitisers (MDI, TMA, and HCPt), and 3 irritants (Phenol, SA, and SDS) suggested that determination of intracellular IL-18 levels in cell line NCTC 2544 correctly identified the skin sensitisers. The irritants and the respiratory sensitisers did not induce changes in IL-18 levels. To confirm this finding, the test was further evaluated using the expanded chemical list that was made available by the GA in October 2008. This list contains pre-/pro-haptens. Table 3 summarises the results of this experiment, while Figure 3 shows examples of typical dose-response curves.

The results confirmed the earlier observation that skin sensitisers induced IL-18 production (accuracy > 99.9%), while respiratory sensitisers and irritants did not. The intra-laboratory reproducibility in terms of accuracy was high.

An inter-laboratory study (including 3 laboratories) assessing the ability of this test to distinguish sensitisers from non-sensitisers and respiratory sensitisers was initiated. The existing SOP was evaluated by the different principal investigators. A refined version of the SOP is now available in the internal database. Extensive standardisation and harmonisation of the various steps of the test was agreed upon by the laboratories involved.

The entire list of chemicals in Table 1 will be covered by this inter-laboratory trial.

Test 2: Assessing the potency of skin sensitisers using a reconstituted human epidermis model with reduction of metabolic activity (viability) as read-out

This assay mimics the penetration of the stratum corneum and general irritation of

Table 2. Down-prioritised activities.

Activity	Reason
DC maturation test Alternative Testing Strategies – Progress Report 2009, Annex 2-4, pp 167-169)	The novelty of the Sens-it-iv tests lies in the fact that MUTZ-3 cells were used. This cell line was identified as the most *in vivo*-like DC available (as compared to monocyte-derived DC). However, extensive efforts could not identify novel maturation markers (or combinations there off). Thus, the test had no added value as compared to the existing DC maturation tests (e.g. U937, h-CLAT), which currently are under prevalidation by ECVAM.
Human reconstituted epidermis test	Research in this area gave a good insight in (i) factors that affect the integrity of the epidermal barrier and (ii) the importance of epidermal integrity for responsive to sensitisers. It was observed that only reconstituted epidermis with good barrier function (reflecting healthy/intact skin) could discriminate between chemical skin (being positive) and respiratory sensitisers (being negative) with as read-out: IL-1, IL-8 or MTT. In contrast, epidermis with poor barrier function (reflecting sick/injured skin) could not discriminate. Development of a test based upon human reconstituted epidermis was not pursued on the basis of the existence (and commercial availability) of numerous similar test systems (e.g., SkinEthic, MatTek, CellSystems). Note: The EST-1000 model from CellSystems was implemented as second tier in a tiered approach (See Tests)
Precision-cut lung slices (PCLS)	The implementation of PCLS on human lung tissue significantly helped to improve or understanding of the modes and mechanisms of action that are relevant for respiratory sensitisation. Until the GA in October 2009, the PCLS approach was considered as the only test available for assessing lung sensitisation (read-out: cytokine profiles). However, no novel markers or marker profiles could be identified using 'omics, primarily due to the complex composition of PCLS (multiple cell types) and considerable donor-to-donor variation. Since novel *in vitro* test systems with good potential are now available (developed outside Sens-it-iv) and are under evaluation by Sens-it-iv (See tests), the PCLS are used primarily for confirmation of identified novel markers and marker profiles identified by 'omics using these new cell-based test systems.

epidermal cells. The great advantage of this model is that chemicals, independent of their solubility in water, may be topically applied on the very same type of filter discs used in human patch testing.

While performing dose-finding experiments, it was previously observed that skin sensitisers (CIN, DNCB, TMTD, and CA), but not respiratory sensitisers (MDI, TMA, and HCPt), penetrate the *in vitro* "stratum corneum" and affect the viability ofkeratinocytes (KCs). The read-out was reduction in metabolic activity (viability), as measured by MTT.

It was confirmed that respiratory sensitisers (MDI, TMA, HCPt, HDI, MA, and GA) are not active in this assay. Unfortunately, the assay was found not to properly differentiate irritants from skin sensitisers. It was hypothesised that selectively applied to skin sensitisers identified by any of the other assays the obtained EC_{50} values (Figure 4) would provide information on the potency of the skin sensitiser. This hypothesis was assessed by exposing an in-house developed epidermal equivalent (rHE) to a selection of skin sensitisers covering the entire potency scale (extreme to weak).

The EC_{50} values were determined and correlated with the EC_3 values determined by the Local Lymph Node Assay (LLNA), which is currently used to classify sensitisers. As show by in Table 4, the EC_{50} was capable of placing the chemicals in the same potency box as the LLNA EC_3.

Two outliers were identified. Glyoxal was classified by the rHE as weak instead of moderate. This misclassification can be explained by the fact the chemical polymerised during the application process. Cinnamic alcohol (CA) was misclassified presumably due to auto-oxidation into cinnamaldehyde (CIN). Both problems were also identified by other groups working with other cell systems. Eliminating these outliers as data points resulted in a cor-

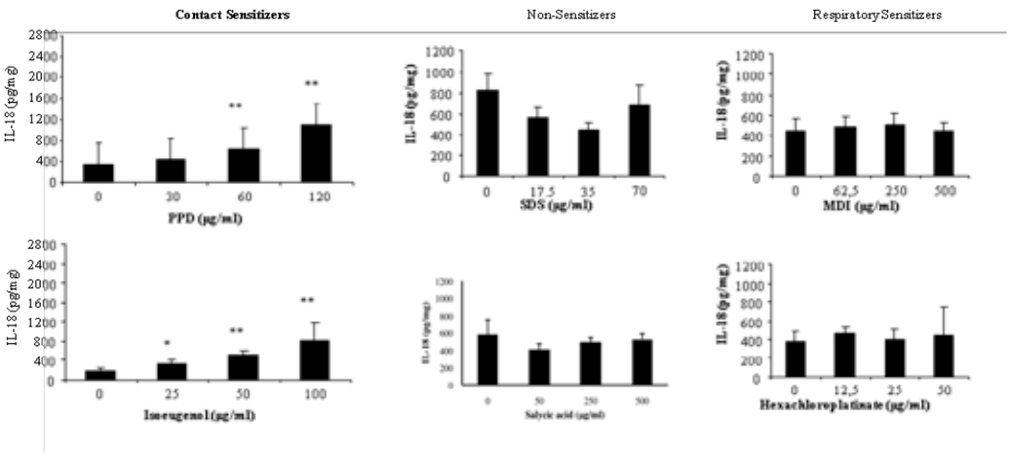


Figure 3. Dose-response curves for selected chemicals.

Table 3. Overview of the results.

IL-18 induction (+)	No IL-18 induction (-)
	Diphenylmethane diisocyanate (MDI)
	Ammonium hexachloroplatinat (HCPt)
2,4-dinitrochlorobenzene (DNCB)	Hexamethylene diisocyanate (HDI) *
Cinnamaldehyde (CIN)	Maleic anhydride (MA) *
Tetramethyl thiuram disulfide (TMTD)	Glutaraldehyde (GA) *
Resorcinol (Res)	Trimellitic anhydride (TMA)
Oxazolone (Oxa) new	
Glyoxal (Glx)	Sodium dodecyl sulphate (SLS)
2-mercaptobenzothiazole (MBT)	Salicylic acid (SA)
4-nitrobenzylbromide (NBB)	Phenol (Ph)
2-bromo-2-(bromomethyl) glutaronitrile (BBGN) new	Glycerol (Gly)
	Lactic acid (Lac)
Isoeugenol (Iso)	Chlorobenzene (CB) *
Eugenol (Eug)	P-hydroxybenzoic acid (PHBA) *
Cinnamic alcohol (CA)	Benzaldehyde (BA) *
	Diethyl phtalate (DEPH) *
Paraphenyldiamine (PPD) (skin sensitizer not part of the chemical list)	Octanoic acid (OA) *

* New since *Progress Report 2009*

relation (r^2) between rHE and LLNA of > 0.9 ($p < 0.01$).

Two limitations of the test were identified. PPD could not be classified as the chemical interferes with the MTT assay. The chemicals TMTD and MBT could not be dissolved at concentrations that were high enough to reach an EC_{50}. Both DMSO and acetone-olive oil were assessed as vehicle to address this issue but without success.

The high correlation observed with chemicals for which EC_{50} could be obtained stimulated the initiation of an inter-laboratory study (including 3 laboratories) assessing the ability of this test to provide data allowing potency labeling of skin sensitisers.

In order to assure the available of rHE, the performance of the in-house rHE, the SkinEthic model and the EST-1000 model from CellSystems was compared. The latter model was found to reproduce the results obtained with the in-house model,

while the SkinEthic model did not. Implementation of the EST-1000 was accomplished. The average of 3 independent experiments, each with duplicate measurements.

The existing SOP was adapted to include the EST-1000 test model, and evaluated by the principal investigators representing each laboratory. A refined version of the SOP is now available in the internal database. Extensive standardisation and harmonisation of the various steps of the test was agreed upon by the laboratories involved.

The entire list of chemicals in Table 1 will be covered by this inter-laboratory trial.

Test 3: Identification of respiratory sensitisers using a human alveolar-endothelial cell based assay with cytokine expression as read-out (new)

This novel test model was developed outside the Sens-it-iv project, but within the Sens-it-iv sphere (Figure 1) by Dr. Iris Hermanns (Universitätsmedzin der Johannes Gutenberg-Universität Mainz, Institut für Pathologie). The test is novel for sensitisation testing, since this is to our knowledge the only test system for the human alveolar compartment of the lung that builds upon human cell lines (H441 and ISO-HAS-1) and that reveals *in vivo*-like functionality (morphology, alveolar type 1 and type 2 cells, reconstituted epithelium with high trans-epithelial resistance (TEER), transport, *in vivo*-like responses to immunomodulators (e.g., LPS)) that is relevant for the purpose of Sens-it-iv. The need for endothelial cells to provide the alveolar cells with appropriate *in vivo*-functionality was demonstrated.

As it was not developed for assessing sensitisation, a first evaluation using the learning set of chemicals was performed. Dose-finding experiments were performed using reduction in TEER as read-out instead of increase in cytotoxicity. Table 5 summarises the results of this pilot experiment and compares them with the cytokine responses in exposed human PCLS.

In general, test systems tests based upon lung tissue or lung cells are more responsive to respiratory sensitisers as compared to skin sensitisers and irritants. As for the skin, IL-8 seems to be able to discriminate both groups of sensitisers, but not between sensitisers and irritants.

On the basis of these promising results, a larger evaluation was initiated, including 3 skin sensitisers (CIN, DNCB, and Eug), 3 chemical respiratory sensitisers (HCPt, MDI, and TMA), 3 proteins (amylase 1, lipase 1, and protease 1), and 2 controls

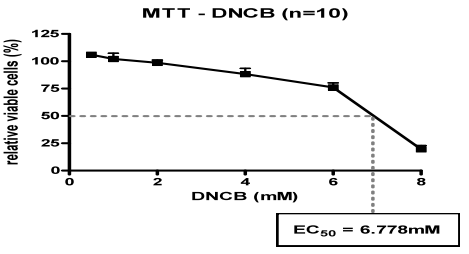


Figure 4. Dose-response curve and EC_{50} calculation for DNCB (10 measurements).

Table 4. Comparison between classification using EC_{50} (rHE) and EC_3 (LLNA).

Decreasing potency	LLNA (EC3%)		rHE (EC_{50} %)	
Extreme <0.1	Oxazolone	(0.01)	√ 4-Nitrobenzylbromide	(0.06)
	4-Nitrobenzylbromide	(0.05)	√ DNCB	(0.14)
	DNCB	(0.08)	√ Oxazolone	(0.34)
Moderate ≥1,<10	Isoeugenol	(1.2)	√ Isoeugenol	(0.39)
	2-Br-2-MGN	(1.3)	√ 2-Br-2-MGN	(0.65)
	x Glyoxal	(1.4)	* Cin. Alcohol	(0.85)
	Cinnamaldehyde	(3.0)	√ Cinnamaldehyde	(1.99)
	Resorcinol	(6.0)	√ Resorcinol	(2.41)
Weak ≥10,≤100	Eugenol	(13.0)	√ Eugenol	(3.92)
	* Cin. alcohol	(21.0)	x Glyoxal	(9.88)

(SA and SLS). The dose-finding experiments using TEER measurements were concluded. Exposure-induced changes in the cytokine profiles were analysed using a cytokine antibody array, with focus on IL-8 and cytokines that were identified by other research groups in the project as potentially useful (e.g., TNF-α, IL-1α, RANTES, MIP-1β, and M-CSF). Data analysis is currently in progress. A draft SOP was established. Supernatants and RNA samples were stored frozen for future 'omics analysis, and the assessment/confirmation of markers identified by other means.

Test 4: Identification of respiratory sensitisers by chronic exposure of a human bronchial EC assay with cytokine expression as read-out (new)

An in-house human bronchial EC model using primary bronchial EC was developed in collaboration with carcinoGENOMICS (Dr Jan Boei, Leiden University Medical Center, Department of Toxicogenetics).

Extensive genomic and functional (reconstituted epithelium with high TEER, CYP activity, responses to immunomodulators, mucus secretion, transport, beating cilia) characterisation revealed that primary cells should be used within passage 4 after isolation from the human lung, and that the cells should be cultivated at an air-liquid interphase. In addition, donor-to-donor variability was described.

In order to address the donor issue and to assure sustainability of the test system, a human bronchial cell line was developed using the telomerase (hTert) approach. The emerging cell line is currently being characterised.

Sens-it-iv identified a commercially-available human bronchial EC model (MucilAIR, Epithelix) with similar characteristics to those of the in-house primary cell model described above. MucilAir has a shelf-life of up to 1 year, assuring sustainability for the duration of the Sens-it-iv project. An additional advantage of this test model is that it allows for both acute and chronic exposure.

As it was not developed for assessing sensitisation, a first evaluation using the learning set of chemicals (MDI, TMA, HCPt, DNCB, CIN, TMTD, SA, and Phe), and the prohapten CA was performed. Dose-finding experiments were performed using reduction in TEER as read-out. Table 6 summarises the results for IL-8 of this pilot experiment and compares them with the IL-8 responses in exposed human PCLS.

The results of the quantification of the inflammatory mediators, before and after the exposure to the chemicals, revealed that each compound has very specific pharmacodynamics on the cytokine. Regarding respiratory sensitisers, even if the profile of IL-8 secreted after 24 hours of exposure is not the same for MDI, TMA and HCPt, it is clear that the secretion increases (2-3x) with increasing (sub-toxic) concentration. With the as-yet unexplained exception if the prohapten CA, dermal sensitisers did not show significant acute

Table 5. Expression of selected cytokines in response to exposure to chemicals.

Compound	Cytokine	Effect on expression	PCLS
TMA	IL-8	+++	+++
	IL-6	-	-
	MCP-1	+++	++
MDI	IL-8	++	+++
	IL-6	++	+
	MCP-1	++	+
HCPt	IL-8	++	+++
	IL-6	++	++
	MCP-1	-	+/-
DNCB	IL-8	+/-	+/-
	IL-6	+/-	+
	MCP-1	-	+
SA	IL-8	-	+/-
	IL-6	-	+
	MCP-1	-	+

(<72 hrs after exposure) effects on IL-8 secretion. Among the irritants tested, Phe stimulated IL-8 release.

Interestingly, analysis of IL-8 responses after chronic (1-3 weeks) exposure to the chemicals revealed a further increase (5-10x) in IL-8 levels upon stimulation with respiratory sensitisers (Figure 5). It was observed that at low concentrations, chronic responses were more likely to occur than acute responses. The chemical haptens did not affect IL-8 secretion, while CA (3x) and Phe (5x) still did.

A detailed SOP is available. Supernatants and RNA samples were stored frozen for future 'omics analysis and the assessment/confirmation of markers identified by other means.

Test 5: Identification of respiratory sensitisers using a BEAS-2B-derived sensitisation marker profile (new)

An intensive but focused genomic analysis of the human bronchial EC line BEAS-2B before and after exposure to selected chemicals from the list approved by the GA in October 2008 allowed the identification of a marker gene profile (35 genes) that seems promising for identification of respiratory sensitisers. The experiment was repeated on the entire list of chemicals (GA in October 2008). This experiment is still ongoing.

Test 6: Identification of sensitisers by exposure of MUTZ-3 (human DC cell line) derived Langerhans cells with cell migration as read-out (further evaluation+refinement)

Previously, a dual-chamber experiment was designed allowing for quantification of the impact of (pre-/pro-)hapten-induced migration of LCs from the epidermis to the dermis. In short, pre-treatment of fluorescently-labeled (CSFE) MUTZ-LC with sensitisers, but not with irritants, induces the expression of a CXCL12 receptor and, hence, enhances migration towards CXCL12. The index of migration CXCL12:CCL5 indicates sensitisers (>1) or non-sensitisers (<1). Of the chemicals tested so far, all sensitisers were reproducibly correctly identified (>99.9% accuracy) (Figure 6).

An inter-laboratory study (including 3 laboratories) assessing the ability of this test to identify specifically sensitisers was initiated. The existing SOP was evaluated by the different principal investigators. A refined version of the SOP is now available in the internal database. Extensive standardisation and harmonisation of the various steps of the test was agreed upon by the laboratories involved.

The entire list of chemicals in Table 1 will be covered by this inter-laboratory trial.

Test 7: Identification of skin sensitisers using a DC-derived sensitisation marker profile (new)

An intensive but focused genomic analy-

sis of MUTZ-3-derived DC before and after exposure to the list of chemicals (the GA in October 2008) allowed the identification of a marker gene profile that identified skin sensitisers (Figure 7). As yet, a marker profile for respiratory sensitisers has not been identified.

Using the 75 genes, all skin sensitisers were reproducibly correctly identified (>99.9% accuracy) (Table 7). On the basis of these positive results, an more extensive experiment was initiated covering the chemicals (including the respiratory sensitisers) listed in Table 1. The data were collected and are currently being analysed. A detailed SOP was made available.

Test 8: The T-cell priming assay for chemicals and proteins (further evaluation & refinement)

The previously reported *in vitro* T-cell priming assay consists of a T-cell amplification step prior to the actual T-cell *in vitro* priming assay. This protocol was optimised to the extent that the amplification step now can be omitted (Figure 8). In addition, the original endpoint (IL-2 production) was replaced by cell proliferation (Figure 9) and detection of TNF-α and IFN-γ (Figure 10).

Test 9: The bio-activation assay (new)

An *in vitro* assay for assessing metabolic activation was developed (Figure 11). When THP-1 cells are exposed to sensitisers haptens, but not pro-haptens, they induce the release of, e.g., IL-8 into the culture medium. Similarly, haptens stimulate both neutrophils and THP-1 in a transwell

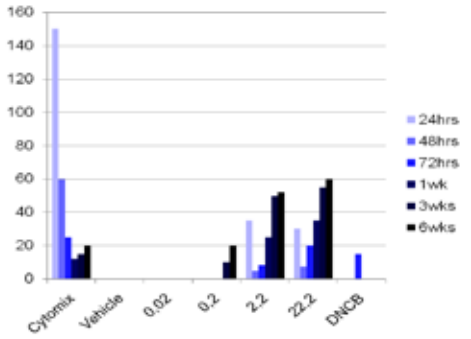


Figure 5. Expression of IL-8 in acute and chronic response to exposure to chemical sensitizers using HCPt and DNCB as examples.

set-up containing both cell types. When pro-haptens are applied on the transwell cell system, only neutrophils react by releasing IL-8. Apparently, the reactivity of the chemical metabolites with cellular protein is so fast that not enough metabolite can diffuse into the lower compartment with the THP1 cells.

This assay set-up was further evaluated using 4 haptens (DNCB, CIN, TMTD, and Res), 6 pre-/pro-haptens (isoeugenol, eugenol, CA, PPD, etc.), and 4 controls (SLS, SA, Phe, and Gly) (Table 1).

The read-outs that will be employed include IL-8, p38 MAPK, and CD markers (immune stimulation), cell viability, glutathione depletion, and Nrf2 (+ related genes) activation (stress responses).

An SOP draft was established. Testing of the chemicals in Table 1 will be initiated.

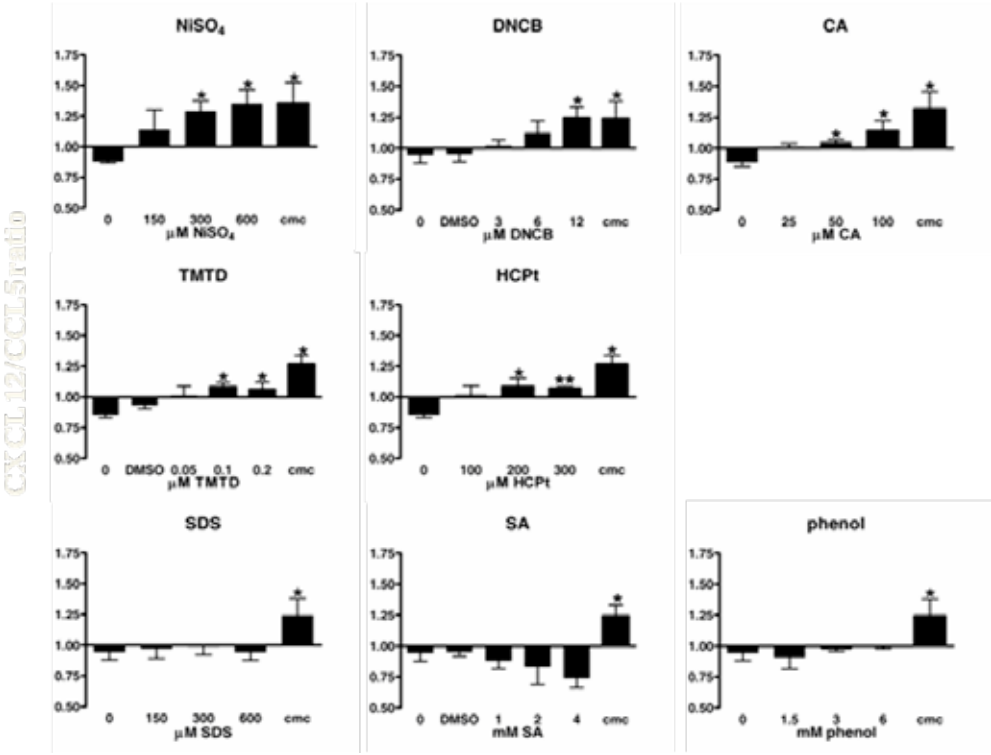


Figure 6: Identification of sensitisers using DC migration. A few examples. $NiSO_4$ is a well characterised skin sensitiser.

D. From Pathways to New Markers

A concerted action employing proteomics, genomics, kinomics, and CD-omics was initiated by the Sens-it-iv GA in May 2008. The cells involved included KC and DC. Lung EC (baseline) data (genomics) were obtained in collaboration with the carcinoGENOMICS project.

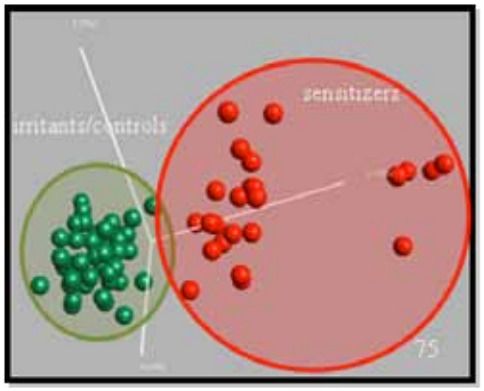
Proteomic analysis of human primary keratinocytes before and after exposure to chemicals approved by the GA in October 2008 (see also Table 3) allowed the identification of proteins that were specific for sensitisers (Figure 12). Currently, this list of proteins (N = 113) is being evaluated with respect to pathways affected for comparison with the genomic data. Soluble proteins for which specific antibodies or an ELISA is commercially available will be prioritised for confirmatory experiments.

Similar experiments were performed on the MUTZ-3 cells. A genomic profile of 75 genes with specificity for skin sensitisers has been identified (*Test 7*). Proteomic analysis has been finalised. Currently, this

Table 7. Overview of the results.

Positive	Negative
2,4-dinitrochlorobenzene (DNCB)	Sodium dodecyl sulphate (SLS)
Cinnamaldehyde (CIN)	Salicylic acid (SA)
Tetramethyl thiuram disulfide (TMTD)	Phenol (Ph)
Resorcinol (Res)	Glycerol (Gly)
Oxazolone (Oxa)	Lactic acid (Lac)
Glyoxal (Glx)	Chlorobenzene (CB)
2-mercaptobenzothiazole (MBT)	P-hydroxybenzoic acid (PHBA)
4-nitrobenzylbromide (NBB)	Benzaldehyde (BA)
2-bromo-2-(bromomethyl) glutaronitrile (BBGN)	Diethyl phtalate (DEPH)
	Octanoic acid (OA)
Isoeugenol (Iso)	
Eugenol (Eug)	
Cinnamic alcohol (CA)	

Figure 7. Principle Component Analysis (PCA) with the 75 most promising genes.



list of proteins (N = 150) is being evaluated with respect to pathways affected for comparison with the genomic data. Soluble proteins for which specific antibodies or an ELISA is commercially available will be prioritised for confirmatory experiments.

E. Scientific Side-Effects

Finding the in vitro *conditions supporting the most* in vivo*-like EC-DC interactions*

A significant breakthrough was made for the keratinocytes (KCs)-DC co-culture. A reconstructed human epidermis model containing CD34-derived langerhans cells

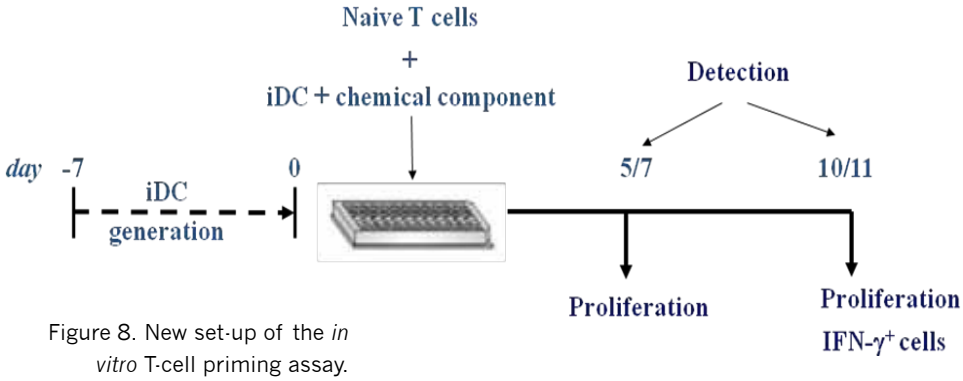


Figure 8. New set-up of the *in vitro* T-cell priming assay.

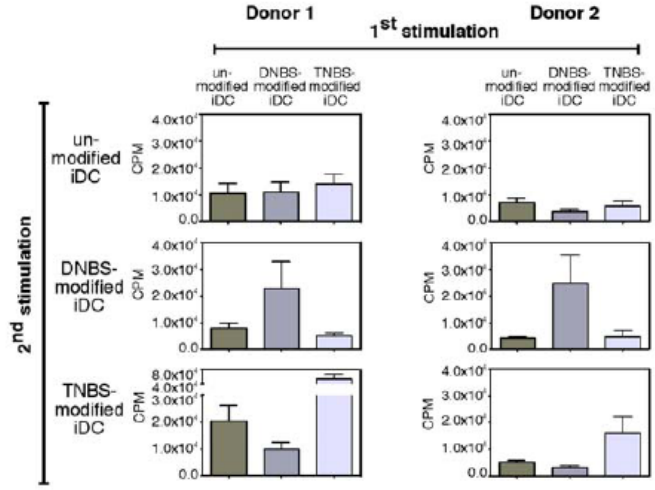


Figure 9. *In vitro* T-cell priming assay exemplified for the skin sensitisers DNBS and TNBS.

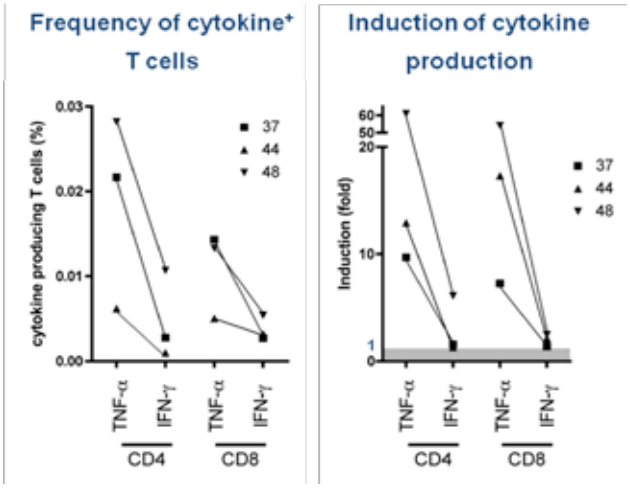


Figure 10. *In vitro* T-cell priming assay: Simultaneous detection TNF-α and IFN-γ.

(LCs) (RHE-LC) responded to sensitisers by induction of LC-specific genes. Sensitiser-specific response patterns were observed. It is therefore an interesting and valuable functional research model for studying case-by-case the effect of chemicals on a LC/KC 3D co-culture model.

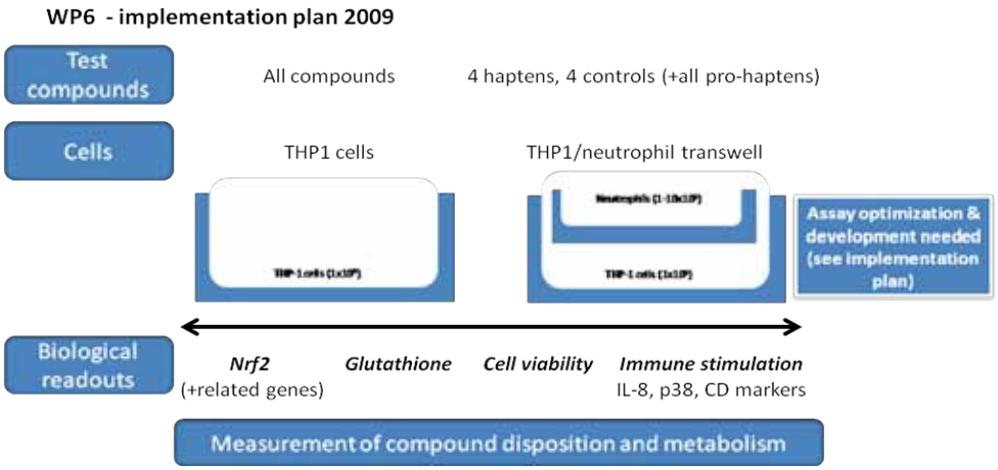
Identification of contact allergens based on innate immune responses

Recent evidence suggests that contact allergens provoke innate immune and stress responses by triggering Pattern Recognition Receptors (PRRs) such as TLR2 and TLR4, and the NLRP3 inflammasome as well as the production of reactive oxygen species (ROS). This underlines the view that the response of the immune system to contact allergens uses the same pathways as the response against microbial infections. However, the mechanisms are different since contact allergens modify proteins and induce tissue damage and DAMPs.

It was now demonstrated that contact allergens such as 2,4,6-trinitrochloro benzene (TNCB) activate innate immune and stress responses by the direct modification of proteins, altering their function and indirectly by the induction of tissue stress and damage resulting in the activation of NADPH oxidases (NOX), and subsequent production of reactive oxygen species (ROS), and in the production of ligands (DAMPs) that activate Toll-like receptors (TLR) and the NLRP3 inflammasome.

This research established the mechanistic understanding of the realtion between skin sensitisers and IL-18 induction (*Test 1*).

Figure 11. Testing strategy for assessing metabolic activation.



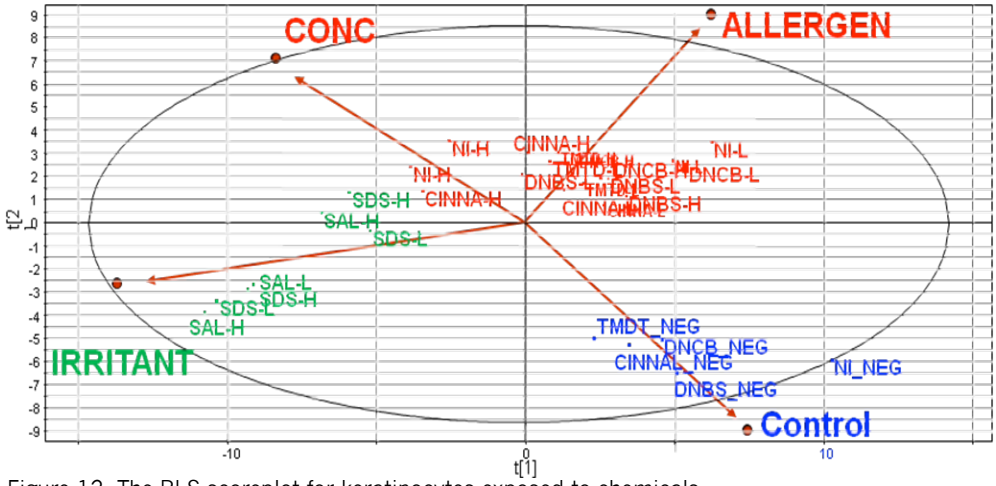


Figure 12. The PLS scoreplot for keratinocytes exposed to chemicals.

F. Dissemination & Technology Transfer

Regular activities—e.g., >40 peer reviewed publications; presentations and sessions at congress and meetings (7th World Congress, SOT, EuroTox, ITCASS, COLIPA, IVTIP, ecopa); website (*Sens-it-iv.eu*); newsletters—in this area were continued. The possibility to organise in 2011 a congress fully dedicated to sensitisation and targeting dissemination of Sens-it-iv knowledge and expertise is currently under consideration.

Technology transfer is currently focusing 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 and the Occupational Safety Round Table Group in the United States, BASF, IVTIP, COLIPA, the Danish *In Vitro* Toxicology Network, and 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.

Concrete progress was made with respect to *Test 1* and *Test 2*, which in a tiered approach are believed to be able to determine the potency of skin sensitisers. Upon request from the Dutch Funding Agency ZonMW to submit a project for prevalidating the tiered approach, the GA unanimously decided based upon the data presented in October 2009 to accept this opportunity. Currently the tiered-testing approach is under prevalidation, involving University of Milano, the VUMC, Hogeschool Utrecht, RIVM, BASF, Novozymes and TNO. ECVAM was included as adviser.

G. Training & Education

Internal practical training on test performance was continued on *ad hoc* basis.

Sens-it-iv organised a Summer school in June 2009 in collaboration with the Hogeschool Utrecht. Currently, the development of an e-learning tool and the organisation of a congress dedicated to Sens-it-iv knowledge and tests are under discussion.

Next Steps

A. Final List of Compounds

Compounds will be distributed upon request.

B. Novelty

Using the database with information about chemicals, proteins, and test systems with relevance for sensitisation, Sens-it-iv will continue to assess the novelty of the emerging approaches and markers to assure that tools are developed that fill out gaps in the currently available toolboxes.

A suggestion as to how Sens-it-iv tools could be integrated the in a tiered-testing scheme is exemplified in Table 8 for skin sensitisation.

C. Tests

Table 9 summarises the status of the various tests that currently populate the Sens-it-iv toolbox. These tools will be further prepared for prevalidation by ECVAM by testing more compounds, and by completing the documentation that is required by ECVAM (test submission form (TSF), SOPs).

During the coming period, the following activities are planned:

- For each test in the box, a TSF will be refined or established if missing.
- SOPs will be updated based upon the results obtained by screening more chemicals (intra-laboratory) or on the basis of experiences from intra-laboratory technology transfer activities.
- Modifications of the TSF and SOPs are based upon intra-laboratory evaluations (E) and inter-laboratory trials (pv) subjecting tests to the compounds of Table 1.
- The most advanced tests will be subjected to inter-laboratory trial assessing transferability and reproducibility (pv), while testing the compounds of Table 1.

Evaluation and prevalidation (including the ZonMW-funded prevalidation exercise) will be performed according to the advice provided by ECVAM.

D. From Pathways to New Markers

For the remaining period, focus will be on the establishment of smaller, more focused gene profiles for both *Test 5* (lung EC) and *Test 7* (MUTZ-3). In-depth analysis of the genomics data will be performed to get an understanding of the pathways and networks that are affected by expo-

sure to the compounds.

Similarly, analysis of MUTZ-3 and KC proteomic data will lead to the identification of pathways and networks relevant for sensitisation. Furthermore, soluble proteins on the list of identified proteins for which simple tests are available (e.g., ELISA) will be pursued, and more chemicals (Table 1) will be tested.

E. Scientific Side-Effects

The study of the EC/DC interactions in 3D-reconstituted models integrating DC or DC lines (Test development). The impact of HA and its metabolites on the phenotype of DC and DC stimulation will be further explored in order to identify new markers related to skin sensitisation.

F. Dissemination & Technology Transfer

With the end of the project approaching, Sens-it-iv will focus during the 6 months extension period on dissemination of tests and knowledge. The focus activities include discussion fora with the respective stakeholders and a dedicated congress in 2011.

G. Education & Training

The development of an e-learning tool will be initiated.

Table 8. A tiered-testing scheme: a proposal for the skin.

Step 1: Chemical point of view	Step 2: Cellular responses
Physico-chemical characteristics (Q)SAR Protein reactivity 1. Protein haptenation SOP 2. Peptide-binding assay (Gerberick et al.) Metabolic activation Neutrophil - THP-1 test Rec human CYP cocktail (Merck et al.)	Tiered approach 1. NCTC+EE DC 1. DC profile 2. DC maturation (H-CLAT, U937 (COLIPA)) 3. DC migration T-cell 1. Primary stimulation test

Orange: Sens-it-iv tests

Table 9. Overview of the Sens-it-iv toolbox.

		N	TSF	SOPs	Status
Keratinocytes	NCTC2544 test	30	X	X refined	pv ongoing
	Human reconstituted skin(1)	30	X	X refined	pv ongoing
Lung EC	Precision cut lung slices	12		X	CT (Table 2)
	Human reconstituted alveolar epithelium	12	X	X	E ongoing
	Human reconstituted bronchial epithelium	12	X	X	E ongoing
	Specific sensitiser profile	12		X	pv ongoing
DC	Specific sensitiser profile	50		X	pv ongoing
	Maturation	12	X	X	Stopped (Table 2)
	Migration	12	X	X refined	pv ongoing
T-cells	Primary T-cell stimulation	6	X	X refined	pv ongoing
Other	Neutrophil · THP-1 metabolisation tests	12		X	E ongoing

N=number of compounds tested; TSF=test submission form; SOP: standard operating procedure; CT: confirmation test; E: evaluation; pv: prevalidation light

(1): Only for potency testing (Table 2)

Orange text indicates changes as compared to *Progress Report 2009*

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carcinoGENOMICS

Development of a high throughput genomics-based test for assessing genotoxic and carcinogenic properties of chemical compounds *in vitro*

Contract number:	LSHB-CT-2006-037712
Project type:	Integrated Project (FP6)
EC contribution:	€ 10 440 000
Starting date:	1 November 2006
Duration:	60 months

Website: http://www.carcinogenomics.eu

Background

carcinoGENOMICS is an Integrated Project conducted within the European Union's 6th Research Framework Programme (FP6), priority area Life Sciences, Genomics and Biotechnology for Health. Its objective is to develop *in vitro* methods for assessing the carcinogenic potential of compounds as an alternative to current rodent bioassays for genotoxicity and carcinogenicity. This proposal refers to the 4th call within FP6 Thematic Priority 1 Life Sciences, Genomics and Biotechnology for Health, which was published on 8 July 2005.

The carcinoGENOMICS project particularly concerns research on the development of new *in vitro* tests to replace animal experimentation. The project specifically aims at developing robust and effective liver-, kidney-, and lung-based *in vitro* methods 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. By adding high-throughput features for capturing the data richness of the genomics designs to be developed in this project, the novel *in vitro* methods delivered by carcinoGENOMICS, will accelerate chemical testing and render it more efficient. 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.

More specifically, it is envisaged that the *in vitro* assays to be developed under carcinoGENOMICS, will outperform the current *in vivo* chromosomal damage assay, which is hampered by its very limited predictiveness and its high rate of false-positive results. In due time, the *in vivo* assay for genotoxicity may therefore completely be replaced, which would be highly beneficial from an animal welfare perspective in the context of REACH.

Further, while it may be foreseen that Europe's regulatory authorities may not likely accept total replacement of the current rodent cancer bioassay by *in vitro* alternatives on short notice, genomics-based *in vitro* screens which will be developed under carcinoGENOMICS may certainly be helpful in prioritising industrial chemicals, as listed by the REACH regulation, for further carcinogenicity studies, thereby reducing the actual number of animals to be used for *in vivo* genotoxicity testing and for the 2-year bioassay for carcinogenicity in rodents.

Objectives

With the aim to develop *in vitro* methods for testing the carcinogenic properties of compounds as an alternative to the chronic rodent bioassays for assessing chemical genotoxicity and carcinogenicity, the carcinoGENOMICS project will address the following science and technology objectives over the 5-year period:

- To develop predictive mechanistic models based on transcriptome- and metabonome-profiling in rat and human primary cells from prioritised target organs (e.g., liver, lung and kidney) in order to discriminate genotoxic from non-genotoxic carcinogens.
- To optimise currently-available cell systems into more robust cellular systems with an extended life-span, stable phenotype, and xenobiotic metabolic competence.
- To explore the suitability of embryonic stem (ES) cells as an alternative source of robust, human-derived cells for assessing target organ-specific carcinogenic actions.
- To discern the biological pathways which, in the predictive models, are essential for class discrimination, and to test the pathway models for predicting carcinogenicity.
- To build an *in silico* model for chemical carcinogenesis of the liver.
- To describe inter-individual differences in the response to carcinogenic challenges.
- To add high-throughput features to developed assays, in order to facilitate the generation of information on genotoxic/carcinogenic properties of chemicals at high speed and low costs.
- To prevalidate developed models according to (newly to be developed) ECVAM guidelines.
- To investigate how the novel genomics-based *in vitro* tests for genotoxicity and carcinogenicity may fit within the EU

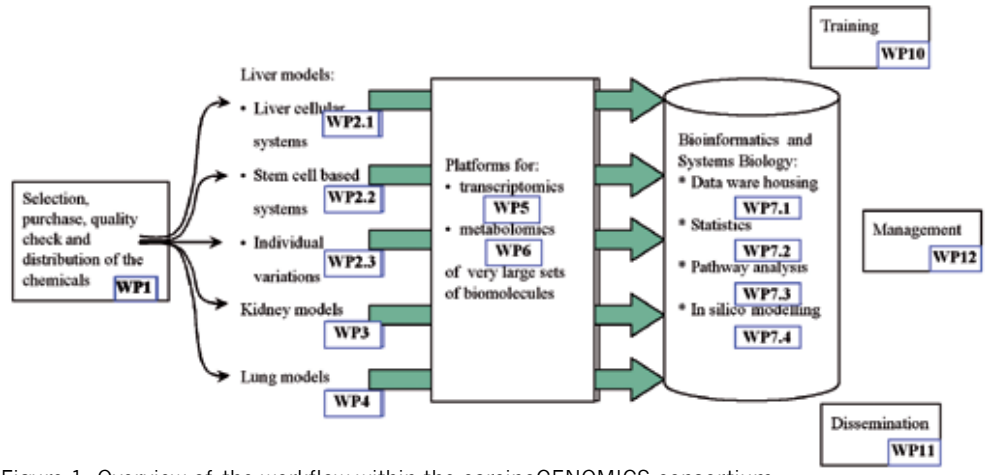

Figure 1. Overview of the workflow within the carcinoGENOMICS consortium.

regulatory and legal frameworks.

- To establish a European public infrastructure giving an integrated view of the genomics data, but also the associated phenotypic information.
- To disseminate the outcome of the study to potential users.

Objectives

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*. For evaluating 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 bio-activation before eliciting their toxic effect. Primary cells, isolated from normal tissue, on the other hand, cope with progressive de-differentiation, which is also associated with loss of their functionality.
- 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.
- The fourth component refers to developing novel, dedicated high-throughput technology for accelerating analysis of genomics responses to human carcinogens *in vitro*, based on discriminative genetic pathways for human genotoxicity and chemical carcinogenesis.

The novel assays will be based on the application of 'omics technologies (i.e., genome-wide transcriptomics and metabolomics) in tests using robust *in vitro* systems (rat/human). A novel human stem cell technology will be explored as well. Genomic responses will be generated from a well-defined set of model compounds for genotoxicity and carcinogenicity. Phenotypic markers will be assessed in order to anchor gene expression modulations and metabolic profiles.

Through extensive biostatistics, literature mining and analysis of molecular expression datasets, the carcinoGENOMICS network will identify differential generic pathways with the capacity to predict mechanisms of chemical carcinogenesis *in vivo*. Transcriptomic and metabonomic data will be integrated into an holistic understanding of systems biology, and then used to build an iterative *in silico* model of chemical carcinogenesis.

The project will proceed in two stages:

- In the first stage, genomics-based *in vitro* screens predictive for genotoxicity and carcinogenicity in target organs (liver, kidney and lung) will be developed, and high-throughput technology will be applied to the assays.
- In the second stage, a limited number of alternative *in vitro* tests will go through the process of prevalidation according to ECVAM's guidelines.

Status

The project has entered its fourth year. Over the first years, much emphasis has been put on the project's choices for cell systems and experimental design, including the selection of model compounds. It is important to note that a characterisation of the experimental models is essential, since it will allow for the establishment of guidelines concerning the 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. In year 3, most attention was put on the processing of results obtained through application of the developed *in vitro* models. Representative *in vitro* models for liver, lung and kidney have been selected, and first challenges of

these models by selected carcinogens and non-carcinogens for generating 'omics responses are nearly completed. With the transcriptomics data obtained after treatments with the chemicals from Phase I, supervised learning methods have been used to build reference compendia, and their robustness was tested by cross-validation. One criterion for the quality of a model was whether such methods can reliably discriminate between the different toxicity classes. Such reference compendia will be used in further steps to classify chemicals from Phase II and/or to extend the reference compendia. Results so far indicate clear possibilities for selecting the most optimal liver and kidney assays. This will be subjected to prevalidation in the next step. It appeared quite difficult to generate an optimal lung model. After considerable trial and error, one model has been selected, but it appeared not yet feasible to transform this into a robust assay. There will therefore be no lung model subjected to prevalidation.

High-throughput features (robotised RNA extraction and labelling, 96-well format for hybridising and scanning microarrays) have been successfully added. The data-warehousing infrastructure is ending its developmental phase and also, major modelling efforts have been undertaken.

Next Steps

The next step will be to make a start with the second stage of the project. Main objective will be that these alternative *in vitro* tests for liver and kidney will go through the process of prevalidation according to ECVAM guidelines. During this prevalidation study, the liver and kidney assays identified as promising will be tested for their transferability and reproducibility. Furthermore, they will be challenged with a new set of compounds. The use of reference compounds that were not included in the "training set" is necessary in order to properly assess the predictive capacity of the methods.

The work on the selection of compounds has been in part published and completed. The chemicals to be used in the prevalidation studies will be identified from among the two agreed lists of chemicals produced during this project. The number of chemicals tested in each selected model needs to be extended. These chemicals will be different from those used in the "training set". In Phase II, an additional set of 15 chemicals will be needed to build up a model, which will allow the classification of genotoxic carcinogens, non-genotoxic carcinogens, and non-carcinogens. Among these 15 chemicals, 3-4 chemicals will be tested in the same assay in 2-3 laboratories in order to evaluate the transferability of the models and their between-laboratory reproducibility. The validation chemicals will be tested under blind conditions during 2010 and 2011. The acquisition, coding, and distribution of testing chemicals to the laboratories will be organised following the prevalidation kick-off meeting.

ECVAM will ensure that lead partners will produce detailed SOPs (standard operat-

ing procedures) for the refined and optimised tests. A detailed study plan will be communicated and approved. The SOPs of the selected test methods will be revised and amended, if necessary. The lead laboratories that have established the promising test methods will be responsible for the technical guidance to the participating laboratories.

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PREDICT-IV

Profiling the toxicity of new drugs: a non animal-based approach integrating toxicodynamics and biokinetics

Contract number:	HEALTH-F5-2007-202222
Project type:	Integrated Project (FP7)
EC contribution:	€ 11 330 907
Starting date:	1 May 2008
Duration:	60 months

Website: http://www.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 per dose group 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 lifestyle 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 can be forged. The integration of systems biology into predictive toxicology will lead to an extension of current knowledge in risk assessment, and will lead 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 preclinical animal testing. Such integrated *in vitro* strategies will successfully result in a reduction of animal experimentation, and thereby decrease the cost and time of lead compound identification.

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

- Compilation and evaluation of available *in vivo* and *in vitro* data to select the relevant pharmaceuticals for an optimised experimental design in the cell culture systems.
- Design of a relational database structure for all *in vivo* and *in vitro* data, and programming of database.
- Optimisation of existing *in vitro* models for the assessment of potential adverse effects of therapeutic candidate compounds in long-term or repeated-dose toxicity.
- Integration of bioavailability/pharmacokinetics into a predictive approach based on *in vitro* data.

Experimental Design

- As a non-animal based approach, PREDICT-IV builds on the knowledge-base delivered by DG RTD FP6 projects. The progress made in pharmacological/toxicological screening and safety pharmacology did not lead only to the input of an extensive list of scientific networks, it also delivered valuable references for the acquisition of *in vivo* data for compound selection (e.g., PredTox/InnoMed).
- Data acquisition and compound selection were further aligned to integrate different technologies used within the project, as well to facilitate a standardised approach for the diverse cell systems used in PREDICT-IV.
- A relational database to support the data acquisition, storage and handling of data and data analysis has been set-up. The system will be expanded and refined as data are acquired and utilised by project partners.
- The toxicity testing strategy is based on a systems biology approach. The endpoints of cellular dysfunction and mechanisms of toxicity will be derived using an integrated approach encompassing transcriptomic profiling, metabolomic profiling, and high-content imaging.
- A novel feature of the experimental design is to determine also the actual exposure levels of cells to drugs and/or their metabolites. Since many compounds bind to plastic, glass and cellular or culture media proteins, and in addition may be unstable or me-

tabolised, the actual concentration of a compound in cell culture media is not necessarily the amount applied. Thus, we will measure the actual concentration of parent compound and its metabolites in supernatant medium and cytosolic extracts at defined time points. This will allow a better comparison between *in vitro* data collected here and existing *in vivo* data.

Results

Compound Selection

Compound selection was accomplished for all cell systems according to the following selection criteria:

- Availability of data pertaining to *in vivo* target organ toxicity (hepatoxicity, nephrotoxicity and neurotoxicity).
- Availability of compounds
- Legal aspects and technical/safety measures.
- Pharmacokinetic information (*in vivo* and *in vitro*).
- Biochemical and biophysical properties.

Cell Culture Models

The optimisation, standardisation and characterisation of the long-term human-based cell culture models utilised for assessing hepatotoxicity, nephrotoxicity and CNS toxicity were finished within the first period of the PREDICT-IV project. Further preliminary tests for all of the selected compounds were started to identify compound concentrations for the subsequent long-term exposure/repeat dose experiments. In parallel, pilot studies were arranged to gain standardised protocols within the work-flow as soon as possible. These studies were conducted in close collaboration with all partners of WP4 to assure a smooth transfer of knowledge and handling between the different work packages.

Long-Term Human Models for Hepatotoxicity

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 various 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.

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: ten 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), multi-drug resistance protein-1(MDR1), multi-drug 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.

Human-Based Cellular Systems for Nephrotoxicity Testing

The cell model has been selected is the RPTEC/TERT1 cell line. These cells form monolayers, become contact-inhibited, and differentiate into a stable phenotype displaying low glycolytic activity. Additionally, this cell line exhibits many characteristics of the human proximal renal tubular cell *in vivo*. RPTEC/TERT1 can be maintained over prolonged periods as tissue monolayers, thus allowing long-term exposure to test compounds.

SOPs have been established for cell culture medium preparation (serum-free, hormonally-defined), cell culture propagation, maintenance, and also differentiation of cells. Characterisation of the cells has shown that differentiation results in a change of major metabolic pathways. Transcriptomic analysis (Illumina platform) of differentiated vs. undifferentiated monolayers demonstrated an over-representation of the following pathways: oxidative phosphorylation, electron transport, and protein biosynthesis.

Dose range-finding experiments for all selected nephrotoxins have been conducted in 96-well plates for 14 days with daily repeat dosing. Additionally, the effect of hypoxia on the toxicity profile of these compounds has been established.

The first of the compounds, Cyclosporine A, has been tested in a full-scale experiment at 5 and 15 µM in differentiated RPTEC/TERT cells cultured on porous supports. Cells were exposed for 1 day, 3 days, and 14 days. Whole genome transcriptomic analysis (WP4.1) revealed a strong time- and dose-effect. The data are currently being analysed in more detail. Metabolomics (NMR and MasSpec) and peptide profiling are being conducted. A perfusion system has been optimised for renal epithelial cells, which allows fully automated repeat-dose treatment.

Non-Target Organ

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 lines, and in HepaRG cells. Analysis of the data is ongoing.

Cellular Systems for CNS Toxicity Testing

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 the *in vitro* BBB model (co-culture of brain capillary endothelial cells and glial cells), the 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 results obtained (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 Vitro Kinetics & Human Kinetic Prediction

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 cell culture systems used, parameters that are essential for each test (drug) item have to be taken into account and controlled. These include:

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

An intensive literature search for analytical techniques of the selected compounds was performed, and the set-up for kinetic measurements of these compounds was started. Using test samples from partners of WP2, an alignment with other work packages was done.

Profiling, Imaging & 'omics

In conjunction with WP2.2 (renal epithelial cells), a series of experiments were conducted in order to optimise harvesting protocols for metabonomic, transcriptomic, and proteomic technologies. Issues such as quenching reagent, washing, cell lysis, cell number, storage and labelling were addressed. Several protocols were tested under different conditions, and the most efficient protocols were selected without any compromise to analysis procedures. SOPs were drawn up and are now available to the consortium.

Next Steps

- Expansion of the studies to all compounds and cell models.
- Generation of the corresponding metabonomic, transcriptomic, and proteomic data.
- Application and measurement of the

corresponding kinetic data.
- Identification of early and late biomarkers of cell stress and dysfunction.
- Identification of biomarkers representing activation of pathways of potential value for chronic disease, e.g., senescence, fibrosis, and cell-cycle inhibition.
- Identification of cell type-specific markers of potential clinical use.

Publications

- Tuschl G, Hrach J, Walter Y, et al. (2009). Serum-free collagen sandwich cultures of adult rat hepatocytes maintain liver-like properties long term: A valuable model for in vitro toxicity and drug-drug interaction studies. *Chem Biol Interact.* 181, 124-37.
- Presentations at the 7th World Congress on Alternatives and Animal Use in the Life Sciences, Rome, 30 August-September 2009, published in ALTEX 26(Special Issue), 58-59:
  - PREDICT-IV a new approach for integrated *in vitro* toxicity testing into the early stages of drug development (Burek C)
  - Cell culture approaches chosen to predict adverse effects of therapeutic compounds (Pfaller W)
  - The crucial role of biokinetics in *in vitro* testing (Testai E)
  - How to integrate *in vitro* PK/PD information for toxicity prediction (Bois FY)
- Presentation at the PlanetxMap Meeting 2009 in Amsterdam: A new screening tool for CYP-induction (Merck, Darmstadt)

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2.4 Computational modelling & estimation techniques

OpenTox

Promotion, development, acceptance and implementation of QSARs (quantitative structure-activity relationships) for toxicology

Contract Number:	HEALTH-F5-2008-200787
Project Type:	Specific Targeted Research Project (FP7)
EC Contribution:	€ 2 975 360
Starting Date:	1 September 2008
Duration:	36 months

Website: http://www.opentox.org

Background

Progress on a well-engineered modernisation of predictive toxicology information technology and interoperability between toxicology systems and resources is urgently required. Progress on interoperability requires a more coordinated approach on standards, progress on the development of public vocabularies and ontologies and standardised computing interfaces. To address these needs, the OpenTox Framework has been developed as an interoperable, extensible modern computing platform supporting international toxicology researcher needs in data management and integration, ontology, model building, validation, and reporting. The design of the Framework has been guided by user requirements, OECD validation principles, REACH regulatory requirements, and best practices in computing engineering and standards. Insights on user requirements in interdisciplinary science, industry application needs in product development and safety assessment, and the effective support of interdisciplinary R&D guide OpenTox developments of sustainable approaches to infrastructure development and management.

The OpenTox Framework provides unified access to toxicological data, (quantitative) structure-activity relationship ((Q)SAR) models, and supporting information. It provides tools for the integration of data from various sources (public and confidential), for the generation and validation of *in silico* models, libraries for the development and integration of new *in silico* algorithms, and validation routines. 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 compo-

nents 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.

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 is designed to support the integration of state-of-the-art *in silico*, cheminformatics, bioinformatics, statistical and data mining, computational chemistry and biology tools, in addition to the automated data management and integration of *in vitro* and *in vivo* toxicology data.

Experimental Design

OpenTox has been designed as a platform-independent collection of components that interact via well-defined interfaces. The preferred form of communication between components is through web services. Initial research defined the essential components of the architecture, approach to data access, schema and management, use of controlled vocabularies and ontologies, web service and communications protocols, and selection and integration of algorithms for predictive modelling. OpenTox provides applications to non-computational specialists, risk assessors, and toxicological experts, in addition to Application Programming Interfaces (APIs) for developers to develop applications. OpenTox actively supports public standards for data representation, interfaces, vocabularies, and ontologies, Open-source approaches to core platform components, and community-based collaboration approaches, so as to progress system interoperability goals and to maximise involvement of different stakeholder groups in developments in as timely a manner as possible.

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 guiding principles in the construction of OpenTox applications are based on the OECD Principles of (Q)SAR Validation[1], satisfying REACH legislation and user requirements, and the additional design principles of interoperability, flexibility, transparency, and extensibility. OpenTox applications are based on a set of distributed, interoperable, extensible OpenTox API-compliant REST web services. The OpenTox approach to ontology allows for efficient mapping of similar and/or complementary data coming from different

[1] OECD Principles for QSAR Validation. *http://ecb.jrc.ec.europa.eu/qsar/background/index.php?c=OECD*

datasets into a unifying structure, having a shared terminology and meaning.

Results

The OpenTox Framework has been implemented as a set of distributed web services that may integrate multiple resources for the construction and application of predictive toxicity models. A key feature of the OpenTox Framework is that it has been designed in a multi-domain-friendly way, which is essential for data- and model-sharing, repeatability, and validation of prediction results. The OpenTox APIs connect multiple distributed web services in an interoperable manner. Based on OpenTox web services, two initial user applications integrating multiple resources were created as part of the initial prototype: a) **ToxPredict**, which predicts and reports on toxicities for endpoints for a user-provided input chemical structure, and b) **ToxCreate**, which builds and validates a predictive toxicity model based on a user-provided input toxicology dataset.

The OpenTox Framework supports rapid application development and extensibility by using well-defined ontologies, allowing simplified communication of data and meaning between individual services. The ToxCreate and ToxPredict applications show the potential impact of the Framework regarding high-quality and consistent structure-activity relationship modelling of REACH-relevant endpoints. The applications have been made available publicly on the web (*OpenTox.org/toxicity-prediction*), providing immediate user access to the applications as they have been developed. User-based testing and reporting provides a mechanism for users to provide feedback on features and requests which can be quickly taken into account in the agile development approach pursued, so as to improve the services offered to users in a timely manner.

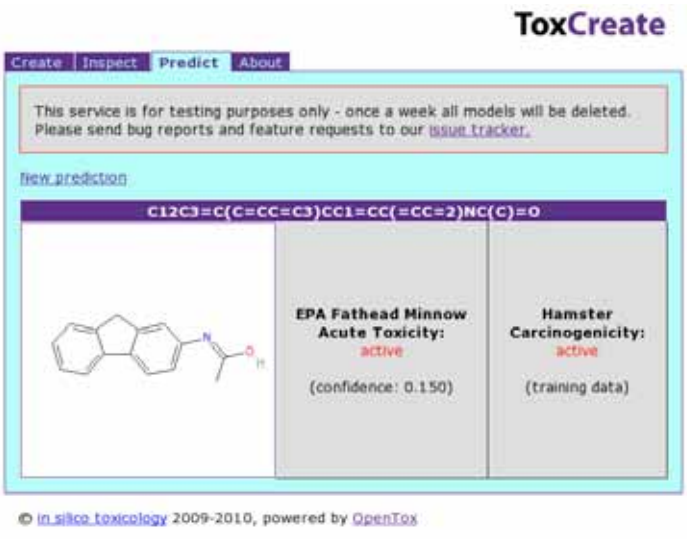
ToxPredict satisfies a common and important situation for a user wishing to evaluate the toxicity of a chemical structure. As illustrated in Figure 1, the user may upload or draw the chemical structure in a web browser and quickly obtain a report back on what current data and model predictions are available for the toxicity endpoints they have interest in. 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 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. The approach offers the potential to be extended to the complete and easy-to-use generation of reporting information on all REACH-relevant endpoints based on existing available scientific research results, and indications when additional experimental work is required, thus satisfying currently unmet industry and regulatory needs.

Figure 1. ToxPredict—OpenTox application for model prediction of toxicity endpoints for chemical structures involving the combination of distributed multiple toxicology resources.

ToxCreate provides a resource to modellers to build soundly-based 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 scientifically sound manner, so as to ensure that the predictive capabilities and limitations of the models can be examined and understood clearly. Models can subsequently be easily made available to other researchers, and combined seamlessly into other applications through the OpenTox Framework.

The initial OpenTox prototype data infrastructure includes ECHA's list of pre-registered substances, 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 have been collected from various public sources (e.g., Chemical Identifier Resolver, ChemIDplus, PubChem) and further checked manually by experts.

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



The applications show the potential impact of OpenTox for high-quality and consistent structure-activity re-

lationship 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.

Challenges

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. In addition to the technical advances of OpenTox in designing a new and modernised computing platform for the field, we are also reaching out to other stakeholders to establish a greater critical mass and collaboration to advance these challenging goals. To this end in 2010 we are also planning to:

- Hold an OpenTox workshop alongside the **AXLR8** coordinating action kick-off meeting in Potsdam, DE on May 30 dedicated to advancing interoperability between international projects, resources and applications.
- Establish a Collaboration Pool and Virtual Organisation supported by OpenTox to develop a more innovative integrated testing strategy for predicting human toxicity risks.
- Host a 1-day workshop ahead of the Euro 2010 QSAR meeting in Rhodes, GR on 19 September to provide guidance and instruction to technical developers so to enable integration of their tools and resources into OpenTox-based applications.
- Organise a Toxicology Ontology Roadmap Forum, hosted by the EBI Industry Forum in Hinxton, UK on 16-17 November 2010 to establish ontology requirements and developments for predictive toxicology, including reuse and extensions to existing biological ontologies for toxicology use cases.

We will continue to actively support public standards such as ToxML for toxicology data representation in the further development of the OpenTox Framework and its APIs.

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 will build and release 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.

Publications

1. Hardy B, Douglas N, Helma C, et al. (2010). Collaborative Development of Predictive Toxicology Applications. *J Chem Informatics* 2, 7.

For more detailed information, please visit the OpenTox Reading Room at *OpenTox.org/home/documents* to download copies of OpenTox presentations and reports.

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2.5 High-throughput techniques

COMICS

Comet assay and cell array for fast and efficient genotoxicity testing

Contract number:	LSHB-CT-2006-037575
Project type:	Specific Teargeted Research Project (FP6)
EC contribution:	€ 3 189 385
Starting date:	1 January 2007
Duration:	36 months

Website: http://comics.vitamib.com

Background

The Comet assay (single cell gel electrophoresis) is a simple, sensitive assay for DNA breaks. It is widely used in genotoxicity testing, as well as in fundamental research in DNA damage and repair, in human biomonitoring, and in ecogenotoxicology. In its standard form, it is labour-intensive and is limited in the number of samples that can be run in one experiment. COMICS was set up to increase the number of samples that can be processed in one run, to improve scoring methods, and to combine cytotoxicity with genotoxicity testing. We anticipate that the Comet assay, in combination with lesion-specific endonucleases, will be accepted as a component in a battery of methods used to test chemicals for potentially harmful effects on human health or on the environment.

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.

Experimental Design

Increasing the Throughput of the Comet Assay

The Comics project has had a dual approach to increasing throughput: (i) superimposing comet analysis on the Cell Array system, established primarily for cytotoxicity testing, with hundreds of minicolonies cultured on a specially prepared glass plate, and (ii) modifying the standard comet assay format by increasing the number of gels.

Modifying the Cell Array: To allow adhesion of cells and agarose, a 3D micropatterned substrate was created, consisting of a covalent agarose layer patterned by deep UV irradiation through a quartz photomask and leading to microwells subsequently treated with extracellular membrane proteins; this patterning favours adhesion of cells, singly or in groups of 2-5 contacting cells. After culture and reaction with chemicals for a few hours, agarose is laid over the array of cell cultures so that lysis and electrophoresis are performed as in the standard assay.

Increasing the number of gels in the standard Comet assay: The comet assay is usually performed on agarose-precoated glass slides, with between 1 and 3 gels per slide; or less commonly on GelBond plastic film, with 6 gels per film. We have increased the number of gels on GelBond film to 48 or 96. In addition, we produced a medium-throughput format, with 12 gels on a normal pre-coated microscope slide, which is

ideal for situations in which individual gels are to be incubated with different chemicals, enzymes, cell extracts, or molecular probes; an incubation chamber with a gasket to ensure separation of the incubations was designed.

Faster Scoring

Automated scoring of comets using IMSTAR Pathfinder instrumentation and software was developed for the new 12-, 48- and 96-gel formats; accurate location of the gels is the critical factor in image capture. Analysis of captured images requires correct identification of comets, rejection of objects that do not meet criteria of shape, size or fluorescence intensity symmetry vs. migration direction, rejection of overlapping comets, and ability to deal with comets over the whole range of levels of damage.

An alternative to conventional scoring is to stain comet head and tail DNA with different colours and then simply to measure the ratio of the two colours. We employed fluorescent in situ hybridisation (FISH) with total genomic DNA tagged with fluorescent marker; hybridisation requires ssDNA, and so fluorescence is mainly from tail DNA. Double-stranded head DNA is counterstained with DAPI. This method is at "proof of principle" stage.

Increasing sensitivity by the use of lesion-specific enzymes

The use of formamidopyrimidine DNA glycosylase (FPG) in combination with the Comet assay to measure 8-oxoguanine is well established. Similarly, endonuclease III detects oxidised pyrimidines, and T4 endonuclease V recognises UV-induced pyrimidine dimers. Uracil DNA glycosylase detects misincorporated uracil. All of these, by converting lesions to breaks, cause an increase in the percent tail DNA in proportion to the amount of damage present. FPG also makes breaks at formamidopyrimidines (ring-opened purines, arising as breakdown products following oxidation or alkylation), at AP-sites (apurinic/apyrimidinic sites, representing base loss, and intermediates in base excision repair) and at alkylated bases (N-7 guanine).

In its basic form, detecting only strand breaks, the comet assay performs poorly in identifying known genotoxic chemicals. However, if a digestion with FPG (following lysis) is included, the effect of the chemicals is greatly amplified. Thus the use of enzymes is likely to enhance the sensitivity of the comet assay as a genotoxicity testing tool. Further trials are needed, especially with non-genotoxins to check that the use of enzymes does not increase the rate of "false positives".

Results

The project was completed in December 2009. Among the deliverables were the following:

- Different Comet assay formats; 12 gels per slide, 48 and 96 gels per GelBond film.

- Cell Array system of mini-colonies on a glass slide, for parallel cytotoxicity and genotoxicity measurement.
- Chamber for incubation of gels (12-gel format) with different reagents or enzymes.
- Coating of slides with novel material to give improved adhesion.
- Calibration of the comet assay.
- Synthesis of chemicals for induction of specific DNA damage
- Characterisation of stains, and development of two-colour fluorescent staining.
- Internal standards; erythrocytes from fish, with small genome, giving comets distinguishable from human comets in same gel.
- Validation trials, testing novel Comet assay formats against conventional format, comparing different scoring methods, and performing dose-response experiments with a range of known genotoxic and non-genotoxic chemicals.
- Detection of specific DNA sequences in comets using padlock probes.
- DNA repair assays, based on oligonucleotide and plasmid arrays, as well as the Comet assay.
- Gene expression kits designed for set of DNA repair genes.
- Commercialisation plan, incorporating equipment, materials and reagents developed during the project.

Next Steps

- Taking two-colour fluorescence staining beyond the "proof-of-principle" stage.
- Using FPG in combination with the Comet assay, against a range of genotoxic and non-genotoxic chemicals, to confirm the increase in sensitivity and to test for the incidence of "false positive" results.
- Developing the padlock probe-based assay for gene-specific DNA repair.
- Commercialisation—a "shopping basket" approach is being adopted by the partner Thistle Scientific, to make our various innovations readily available to comet assay users.

Publications

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NanoTEST

Alternative testing strategies for the assessment of the toxicological profile of nanoparticles used in medical diagnostics

Contract number:	HEALTH-F5-2007-201335
Project type:	Specific Targeted Research Project (FP7)
EC contribution:	€ 3 933 271
Starting date:	1 April 2008
Duration:	42 months

Website: http://www.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. 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 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.

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 es-

sential 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 and small organ fragments (*ex vivo*) 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 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 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.

Experimental Design

Name of the Test Method

Various *in vitro* and *in silico* methodologies to assess the risk of NPs. We will study barrier transport and to assess cytotoxicity, oxidative stress, inflammation, immunotoxicity and genotoxicity. Among other methods, we will use Fe uptake, MTT test, ROS production, LDH assay, WST-1 Assay, ELISAs for cytokines and growth factors, Propidium iodide, HE, DCFDA, mBBr, growth activity test, plating efficiency, Comet assay, *in vitro* base excision repair assay, *In Vitro* Micronucleus Test (OECD 487). Results will be pre-validated in an *in vivo* model.

Clinical Endpoint

Endpoints related to oxidative stress, inflammation, immunotoxicity, and genotoxicity (carcinogenicity).

Cell (Line)

We use cell culture and small organ fragments (*ex vivo*) derived from different biological systems: blood, vascular system, liver, kidney, lung, placenta, digestive, renal and central nervous systems. Human and mammalian cell lines and primary cultures will be used: human endothelial cells, LN229, CaCO2, BeWo, 16HBE, A549, human lymphocytes, rat Kupffer cells, rat liver sinusoidal endothelial cells (LSEC) and hepatocytes, etc. A panel of *in vitro* cell lines will be suggested at the end of the project for toxicity testing of NPs.

Method Description

Most of the assays for NPs toxicity have been modified or developed. The *In Vitro* Micronucleus Test will be performed following OECD test guideline 487, and the latest high throughput modifications of the comet assay developed within the COMICS project are being used.

SOP: The aim of the project is to develop a set of SOPs. Master SOPs at least for two assays for each type of toxicity has been provided.

Endpoints: The project will define the most appropriate endpoints, specific to toxicity of NPs (see above).

How is a positive result defined? Criteria used to classify positive (and negative) results, e.g., cut-off values, profile of relevant markers significantly different from negative control values.

How is a positive result expressed? The response of cells (marker value change) is significantly different (P less than 0.05) from negative control. Units vary according to the test employed.

Applicability

NanoTEST aims to test engineered NPs used in medical diagnostics, but tests developed will be applicable for all natural and engineered NPs, including the following: a) titanium dioxide as benchmark; b) paramagnetic metal oxides which are applied for contrast enhancement as well as for cancer treatment in various biomedical applications; Fe_3O_4, uncoated and coated with oleic acid, c) Silica nanoparticles, (which are defined as promising within tumour targeting, drug delivery and medical imaging and sensing); and d) polymeric materials utilised for the delivery of macromolecules (polylactic glycolic acid, PLGA, to be used as controls in order to ascertain the behaviour of NPs at the biological level

Positive control: The choice of appropriate positive control is very important for conventional chemicals but even more for NPs. As the benchmark for all assays, titanium dioxide will be used. For assays detecting cytotoxicity, oxidative stress and genotoxicity (Comet assay) hydrogen peroxide is used additionally to TiO_2. Certain tests have specific recommended positive control substances. Positively charged dendrimers are being discussed to be used as positive control.

Negative control: Cells cultivated in medium in parallel with cells in medium with test-NP or positive control. Endorem (iron oxide coated with dextran), which is already in use in medicine, will be used as negative control.

Performance: The aim is to develop assays with high sensitivity, specificity and accuracy, giving clear positive and negative predictive value. Several tests have been identified as suitable for nanoparticle toxicity testing. However, some tests appeared to have certain limitations. Work is in progress.

Can the test method be used in a regulatory safety context? Yes. The outcome of the project is to deliver a battery of assays that can be applied to fulfill regulatory requirements (REACH). The project will deliver strategies and a battery of assay methods for testing of NPs. The outcomes of the project will also help to decide whether new regulations are needed for risk assessment of NPs.

Which "R" would the test method impact? We will develop alternative testing strategy for assessment of toxicological profile of NPs. This will have impact on refinement and reduction of animals for toxicity testing of nanoparticles used in medical diagnostic. However, the development of these methods and strategies can be applied also for the assessment of health effects of NPs used and applied in other areas (cosmetic, etc.) and thus can have impact on all 3Rs (Replacement, Reduction and Refinement).

How can the test be used? We will develop an alternative/integrated testing strategy for hazard identification and risk assessment of NPs and thus to suggest combination of the most appropriate assays and methods.

Results

WP1: Characterisation

Status: A detailed chemical and physical characterisation of selected nanoparticles has been performed. Their behaviour in various biological media has also been investigated, obtaining data about size distribution and tendency to agglomeration and precipitation very useful for estimating their potential interaction with living system. New dispersion protocols for the application of nano-sized titanium dioxide to *in vitro* toxicological experiments have been developed. PLGA and iron oxide both coated and uncoated have been characterised.

Problems: Fluorsecence silica nanoparticles ordered from US company appeared to be degraded and thus can not be used in *in vitro* and in *in vivo* studies.

Solution: New silica nanoparticles were offered by depository of JRC and are now under characterisation. As time is limited, only one size NPs (20nm) will be characterised and used for additional studies.

WP2: In Vitro Assays

Status

WP2.0 SOP: As initially proposed, the data sheet for SOP has been updated regularly dependent on the information received from the different partners.

WP2.1 Blood: The experiments on blood-derived cells have been started and the effects of the available nanoparticles have been studied. Immunotoxicity and genotoxicity (both micronucleus as well as the Comet assay) have been studied.

WP2.2 Vascular system: As initially proposed, the interactions of the nanoparticles selected, characterised and available during this 2nd year have been evaluated for cytotoxicity, production of ROS, transport across endothelial cell layers, and genotoxicity.

WP2.3 Liver: As model cell system Hepatocytes, Kupffer cells and Liver Sinusoidal Endothelial cells (LSEC) from rat have been treated with TiO_2, PLGA and silica NPs, cytotoxicity (MTT assay) ROS production (using Carboxy-H_2DCFDA), genotoxicity (Comet assay) have been studied. Internalisation of nanoparticles was studied using immunoflurescence microscopy. NP localisation was determined by image analysis.

WP2.4 Lung: The cytotoxicity of TiO_2 P25 NP as well as PLGA-PEO, coated iron oxide and naked iron oxide was evaluated on human bronchial epithelial cells (16HBE cell line) and human alveolar type II cells (A549 cell line) by measuring the metabolic activity of the cultures (WST-1 assay) and the membrane integrity by propidium iodide (PI) incorporation. The interaction between NPs and WST-1 reagent or PI was tested before cytotoxicity testing. The induction of oxidative stress by TiO_2 as well as PLGA-PEO NPs was measured in the two cell lines by detecting dihydroethidium oxidation using flow cytometry, and thiol depletion was measured using the mono-BromoBimane test using flow cytometry or a fluorescent plate reader. Detection of DHE by a fluorescent plate reader was not possible. Furthermore, the endocytosis of the different NPs by 16HBE and A549 cells was studied using flow cytometry, and the endocytic pathways involved in TiO_2 uptake were evaluated by using specific inhibitors after testing their specificity and efficiency using fluorescent positive controls. To evaluate the translocation of NPs across the respiratory epithelial barrier, different culture conditions and cell lines were tested for their ability to establish a junctional epithelium on two compartment chambers before quantifying the transcytosis of TiO_2 NPs. The induction of an inflammatory response in the 16HBE cell line and A549 cell line was studied by measuring the release of cytokines (GM-CSF, IL-6, IL-8) by ELISA after treatment with TiO_2 P25 NP, as well as PLGA-PEO at non-cytotoxic concentrations. The signalling pathways involved in the inflammatory response were determined by using different inhibitors of kinases. The interference of NPs with cytokine detection by ELISA was also addressed.

WP2.5 Placenta: NP toxicity studies utilis-

ing BeWo cells: Nanoparticle interference testing and toxicity screening have been performed for TiO_2, PLGA-PEO, Fe_3O_4 (naked) and Fe_3O_4 (coated) nanoparticles using the LDH, IL-6 ELISA and WST-1 assays with the placental BeWo cell line. Further toxicity assays (ROS and MTT) have been carried out in the BeWo cells for PLGA-PEO, TiO_2 and Fluoresbrite nanoparticles (with the addition of WST-1 assays for the Fluoresbrite particles also). We found evidence of nanoparticle interference in the LDH assay (with TiO_2 and coated Fe_3O_4), the IL-6 assay (with uncoated Fe_3O_4) and the WST-1 assay (with TiO_2), which emphasises the importance of ensuring that adequate controls are included in the assays. Optimisation of the Comet and MNi assays using BeWo cells is in progress. Further development work will be undertaken using TiO_2 NP in the first instance. Growth rates and exposure to cytochalasin B were investigated in preliminary studies to determine optimal cell seeding conditions. Papers are in preparation presenting the particle interference data and the toxicity data.

BeWo NP transport model development: BeWo model development for use in nanoparticle transport experiments has been completed and validated using Fluoresbrite nanoparticles. A publication reporting these results is in preparation.

Perfusion NP transport model: Preliminary data for transport of Fluoresbrite have been obtained by performing a fetal-side perfusion in Krebs-Ringer buffer containing albumin as a means to assess accumulation of the particles in cotyledon tissue. Additionally, a preliminary placental perfusion with TiO2 has been completed. An SOP was established for both perfusion and BeWo placental models.

WP2.6 Digestive system: As initially proposed, the toxicity of TiO_2, PLGA-PEO, Fe_3O_4 (naked) and Fe_3O_4 (coated) NPs has been evaluated in two different gastrointestinal models, CacoReady™ and Caco-Goblet cells. Toxicity has been evaluated using a Lucifer Yellow permeability assay to assess the integrity of the gastrointestinal barrier in the presence of each nanoparticle. As expected, only TiO_2 NPs were toxic for both models.

WP2.7 CNS: As initially proposed, the evaluation of the effects on cells of nanoparticles selected, characterised and available was a deliverable of the 1st year. Due to delays in the acquisition of the nanoparticles, this deliverable could not be reached during the 1st year, but has been done for M18, and is now delivered.

WP2.8 Kidney: The initiation of the study of the effects of nanoparticles on kidney-derived cells, a 2nd deliverable, has been postponed to M30, due to the delays in the acquisition of the complete set of nanoparticles.

WP 2.9 Assay automation: A case-by-case study has been performed on all the assays proposed by the NanoTEST project and their applicability to automation on our high-throughput sceerning and/or high-content analysis (HCA) platforms has been assessed. Oxidative stress and genotoxicity were selected as end-points, and

among these assays the ROSmw Dihydroethidium oxidation test was selected to be automated on both platforms, and the micronucleus assay on the HCA platform using automated fluorescence microscopy. Automation of the selected assays is still ongoing.

The final test selection was delayed due to the fact that the final assay proposals were distributed during the end of the second year of the project. Also the delays in the characterisation and problems with certain dispersion protocols have delayed the process of assay automation.

Cross-cutting topics: Barrier transport, oxidative stress, immunotoxicity and genotoxicity have been studied cross the cell systems and different models representing 8 tissue and organ systems. The same methodologies for all these endpoints and SOPs are being developed.

Problems and solutions

The main problem in WP2 was the delay in obtaining and characterising the selected nanoparticles, a task of WP1, which forced us to reorganise our list and order of deliverables. The solution has been to rapidly select and characterise the still-missing control nanoparticles, and reorganise the deliverables in order to reach our goals at the end of the project.

We also faced some technical problems due to interaction of the NPs with reagents used for the assays or the secreted proteins (WST-1, PI, LDH, ELISA), and the techniques had to be adapted or replaced and necessary controls added.

In WP2.5, the major challenge that we face is the lack of resources available to deploy for the rest of the project as a result of delays in selection and distribution of nanoparticles. As a result of budget constraints, we were already facing limits on personnel availability for the project and this has been aggravated further. In the absence of further funding, we are extremely limited with regard to the experimental work that can be undertaken during the remaining time on the project. It is therefore not possible for us to evaluate the full range of particles or assays.
A further consequence of the delays/restrictions on particle supply has been that the transport cross-cutting topic has not been able to progress as much as we would have liked. Focus has been on addressing toxicity aspects, and transport studies have not been implemented uniformly across the consortium due to technical limitations on particle detection for the various types of nanoparticles. To solve this, we are trying to attract additional resources to the project locally. This will enable us to continue with some of the work. However, we will have to focus on some clear-cut priorities in order to deploy our very limited resources as effectively as possible.

WP3: In Vivo

Status: In the second half of 2009 we gained ethics committee approval and permission to purchase animals from the Czech Republic. Recently we obtained 30 female Wistar rats with weight 200 ± 20 g

to perform the acute toxicity study. After one week of adaptation, we injected the first doses of oleic acid coated magnetite and titanium dioxide to the animals. After thorough research of the literature we decided to start with 175 mg of nanoparticle/kg. The experiment is now ongoing.

Problems: Reason for later start was delay in selection and characterisation of nanoparticles and consequently slight delay in WP2.

Solutions: The delay in *in vivo* study does not influence overall progress. *In vivo* study is still manageable in the lifetime of the project and can be finished in time.

WP4: Structure-Activity & PBPK Modelling

PBPK modelling: In the period of reference, NanoTEST consortium partner Demokritos worked on Task 4.2 of WP4, that is on the PBPK modelling. More specifically, work has been undertaken to respond to the needs of stage 1 of Task 4.2, the aim of which was to formulate a simple compartment-based model to describe the biodistribution of nanoparticles. Consortium partner IOM provided us with an existing code, written in MATLAB, which describes the progress over time of the retention of particles and the alveolar macrophage (AM)-mediated clearance process in the pulmonary region, as well as the time course of the build-up of the dose after exposure[1]. After being re-codified in FORTRAN language and tested, the clearance-retention model was combined with our lung deposition code (available in FORTRAN), which deals with particle transport and deposition within the regions of the respiratory tract using a numerical model based on an Eulerian approach describing the air flow and aerosol dynamics in the respiratory tract[2]. The outcome of this study is a powerful mathematical tool (in the form of interconnected FORTRAN modules) permitting the relation of inhalation exposure with internally-delivered biological doses. Details of the aforementioned work can be found in NanoTEST deliverable D.4.2 "Completion of a simple, compartment-based PBPK model".

Structure Activity: Report on the development of structure-activity models for toxicity predictions:

With our current work, we (JRC) are trying to demonstrate that one way to identify oxide nanoparticle cytotoxicity via oxidative stress is by looking at the ability of these materials to perturb the overall intracellular redox state. By comparing the redox potentials of intracellular reactions with the material's energy band structure, we have shown that one could identify possible harmful materials on the basis of their potential to generate ROS or affect the intracellular concentration of antioxidants. This theoretical framework provides a tool for designing *in vitro* cytotoxicity assays with oxide nanoparticles and interpreting their outcome in terms of mechanistic chemistry.

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In vitro models have shown a clear link between oxide nanoparticle exposure and the generation of oxidative stress. Under physiological conditions, cells maintain a reduced intracellular state. This process is the result of a balance between the levels of oxidised and reduced species present in a cell. Oxide nanoparticles can create an imbalance in this state, e.g., by acting like catalysts and providing an alternative pathway for the electron transfer process in aqueous redox reactions. The electron transfer occurs at the interface between the oxide and the aqueous species and its feasibility can be evaluated by comparing the energy structure of the oxide and the adsorbed reactants. We compare the calculated conduction and valence band edge positions of 70 oxides with the standard potentials of redox couples active in biological media. The valence band edge of these materials is more than 1 eV below the redox potentials of biological reactions, but for some of them, e.g., CuO and Cu_2O, the conduction band is close to that range, indicating that these oxides are potential catalysts capable of decreasing the reducing capacity of a cell.

In general, the overall picture yielded by our model seems consistent with *in vitro* findings. However, when interpreting *in vitro* cytotoxicity results in the light of this theoretical framework, one should consider that, for small enough particles, the energy band structure changes as the material's size decreases: weakened (i.e., deformed but intact) and dangling (i.e., unsaturated) bonds increase on the oxide surface and, consequently, the energy band diagramme will be affected showing a band tailing effect near the band edges (due to the bond weakening) and/or producing deeper states which arise from the dangling bonds. These events affect the overall electronic structure of the material's surface, as well as properties like the ionisation potential, the electron affinity, the charge density and the chemical potential, finally leading to a higher or lower activity compared to the bulk material. Moreover, nanoparticle injury can also proceed by non-oxidant paradigms. One example is the ability of nanoparticles to dynamically adsorb proteins on their surface. As a consequence of this binding, some particles change their adsorption, distribution, metabolic and elimination (ADME) profiles, showing different potencies or mechanism of action when compared to naked particles. In light of these considerations, we are trying to complete the model by performing studies on the change of physicochemical property values in relation to particle's size and on protein adsorption on the oxide's surface.

Next Steps

WP1: The next step will be the development and application of new analytical methods for the identification and quantification of the selected nanoparticles in biological tissues coming from *in vitro* and *ex vivo* testing.

WP2: In general, we will continue the process of testing the nanoparticles, especially to include the new silica. Specifically, next steps will be:

- WP2.5 Prioritisation of assays and nanoparticles during remainder of project.
- WP2.6 Repetition of some assays in the presence of culture media instead of HBSS buffer.
- WP 2.9 The systematic assessment of the reliability and performance of the selected assays on the automated platforms will be performed.

WP3: We will finalise acute toxicity studies with iron oxide NPs and TiO_2. After we determine the LD_{50} for intravenous injection of the above mentioned nanoparticles, we will have to decide the three doses for magnetite and the one dose for titanium dioxide as benchmark for the *in vivo* nanotoxicity experiment, which we are planning to perform in the autumn (2010). For the decision about the doses we will consult the whole consortium of the project.

WP4: Consortium partner Demokritos has already started working on the second stage of Task 4.2, i.e., the employment of computational fluid-particle dynamics to analyse the behaviour of nanoparticles in the respiratory and cardiovascular system. In relation to Task 4.3, report on the development of structure-activity models for toxicity predictions, we are planning to validate our model against data originated inside the NanoTEST project. We have also already started to implement additional features to the model in order to take into account the quantum size effect and the adsorption of proteins.

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2.6 Forums & workshops

ForInViTox

Forum for research and regulators to meet manufacturers of toxicology methods

Contract number:	LSSB-CT-2007-037779
Project type:	Specific Support Action (FP6)
EC contribution:	€ 288 850
Starting date:	1 September 2007
Duration:	24 months

Website: http://www.forinvitox.org

Objectives

Research on *in vitro* replacement tests by researchers and industry, with substantial support by the European Commission, has resulted in an important number of scientifically sound methods and new strategies. However, the transfer of these inventions to potential users has been much slower than expected, mainly due to difficulties encountered in the transferability, official approval as well as production of test kits under conditions of regulatory requirements. The present gap between inventions and potential users needs to be bridged.

The ForInViTox project, supported by the European Commission within the 6th Framework Programme (FP6), had the purpose to facilitate the development and increase the number of commercially available *in vitro* methods by identifying the most urgent testing needs and the ways to respond to them.

Experimental Design

The German and Spanish national platforms of alternatives (SET and REMA, respectively) investigated the development stage of the methods evolved from the FP6 projects. They found a number of interesting methods that need to be further transferred to become applicable for the end users.

The *In Vitro* Testing Industrial Platform (IVTIP) made an analysis among their members of the needs of the users. There is a need for more mechanistic and physiologically

relevant models. It is also important that the methods are robust and reproducible.

Expertrådet made an inventory of the manufacturers of *in vitro* tests. It showed that their major customers are from the pharmaceutical industry. A serious problem for the technology transfer of the methods is the lack of intra- and inter-laboratory reproducibility.

The reports were summarised and discussed with experts representing the stakeholder groups. This round table meeting, arranged by Silverdal Science Park, came to the conclusion that the most important issues to improve the technology transfer of *in vitro* tests are to:

- Bridge the gap between inventors and end users.
- Improve the development process of *in vitro* models.
- Invent and use instruments to facilitate the transfer.

A "marketplace", The Forum Event, for inventions, applications and products of alternative tests was arranged for the first time in Europe. Aspects highlighted at the Expert meeting were presented in short lectures and then discussed with all participants.

The Forum Event was a joint event of the EU-projects ForInViTox and InViToPharma and IVTIP and organised by Silverdal Science Park. 30 models with commercial potential were presented at the Forum. 17 of them were presented orally and then discussed with focus on commercial applicability, first by a panel and then by the whole audience.

The results from the project are summarised in the "White Book" that comprise:

- A guide for the European Commission on how to promote the transfer of alternative tests to applicable products for the end-users.
- A guide for the inventors of alternative test in the process of transferring a research method into an applicable test.
- A guide for the end users about present and upcoming methods within the area of alternative tests.

Results

The following issues were identified as the most important to improve the technology transfer of *in vitro* tests:

- Identification of the gap between the inventors and the final users
- Bridging the gap
- The development process
- Instruments to facilitate the transfer.

Identification of the Gaps Between the Inventors and the Final Users

Gaps include:

- Intellectual
- Cultural
- Financial

- Legal (concerning dissemination of results).

Bridging the Gap

To be able to bridge the identified gap there is a need of:

- Intellectual and cultural bridges like brokers, meetings and networking; ForInViTox could be one of the bridges.
- Financial bridges like EC or national calls specified for verification of methods as well as other steps of the transfer process; collaboration with final users and CROs.

The Development Process

The following parts are essential for the transformation of an invention to a commercially applicable test method:

- Transformation to a less complex method
- Robustness
- GLP
- Validation
- Different competences for the different steps of the process
- Early involvement of regulators.

FP Projects

- Increased investment in the project management by training, recruitment and a larger portion of the project budget.
- Dissemination of the results after finalising the project.

Instruments to Facilitate the Transfer

- Clear guidance and criteria for validation.
- Showing good and bad examples.
- "Cookbook" for the steps of the transfer process.
- Matching inventions with applications.

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InViToPharma

Workshop on the need of *in vitro* toxicity tests within the pharmaceutical industry

Contract number:	LHSB-CT-2007-037814
Project type:	Specific Support Action (FP6)
EC contribution:	€ 578 000
Starting date:	1 November 2007
Duration:	24 months

Website: http://www.forinvitox.org/invitopharma

Background

A workshop where representatives from manufacturers of toxicity tests, researchers developing new *in vitro* pharmaceutical toxicity tests, the European pharmaceutical industry, and regulatory authorities participate makes it possible for the stakeholders to discuss the supply and demand for *in vitro* pharmaceutical toxicity tests, both new and already existing. The workshop will also increase the business opportunities for the manufacturers of *in vitro* pharmaceutical toxicity tests, often SMEs, as the participants will get plenty of time to sit down and discuss in smaller groups and person-to-person.

Objectives

A dialogue between the stakeholders within the area of pharmaceutical toxicity testing is crucial to achieve a more general use of alternative toxicity tests. More efficient toxicity tests are also an important factor to make it possible for the European pharmaceutical industry to increase its competitiveness. The increasing amount of substances passing the drug development process needs to be tested with efficient methods as early as possible in the process to new pharmaceuticals. That demands more robust and cost efficient methods, not always available today.
The main objectives of this project are:

- To identify the need within the pharmaceutical industry for *in vitro* toxicity tests, e.g., what kind of tests are required, which toxicity areas are of most concern for development of new *in vitro* toxicity tests, high-throughput screening tests, tests moni-

toring the toxicological mechanism in detail, etc.
- To identify and present academic model systems suitable to use for the pharmaceutical industry.
- To identify and present *in vitro* toxicity tests manufactured by enterprises.
- To analyse the correspondence between the available *in vitro* toxicity tests and the test strategies used in the pharmaceutical industry.

Experimental Design

The project started with a general analysis of the current situation after a planning meeting with the Advisory committee with representatives from the stakeholders. The information that formed the base for the analysis was retrieved through questionnaires to pharmaceutical companies.

A 2-days workshop on the subject "What is the need of *in vitro* toxicity tests within the pharmaceutical industry" was held at Silverdal Science Park, Stockholm, Sweden. The workshop consisted both of introductory lectures and smaller working groups focussed on different issues defined after the general analysis described in WP1. The outcome from the working group meetings was reported at a session for all participants followed by a general discussion. A final report from the Workshop has been produced and distributed.

Results

The following inventories took place during first 12 month of the project:

- A general analysis of the supply of knowledge and products that can be used within the preclinical safety testing and the corresponding demand for efficient and robust *in vitro* tests from the European pharmaceutical industry.
- Report summarising the current situation.
- Establishment of collaborations with "New Safe Medicines Faster" initiative, the EXERA consortium, and other relevant organisations.

The InViToPharma and ForInViTox projects cooperated to make the Forum Event—the First Market Place in Europe for inventions—as successful as possible. The Event took place between 12-14 May 2009 at the Karolinska Institute in Stockholm. The meeting was a joint event of the EU-projects ForInViTox and InViToPharma, IVTIP, and organised by Silverdal Science Park, Stockholm, Sweden.

The aim of the Forum Event was to establish a meeting place for representatives from manufacturers of *in vitro* toxicity tests, research projects developing and validating alternative tests, regulatory agencies and end users, for open discussions in order to respond to the increasing testing needs. To distinguish the Forum Event from other research meetings and Congresses, the focus was on application

and applicability of *in vitro* methods, as well as the validation procedure of new test systems. The research methods that will be presented must be in the front of being transformed into a future *in vitro* test and having a commercial potential.

InViToPharma has also been involved in the production of the ForInViTox "White Book".

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START-UP

Scientific and technological issues in 3Rs alternatives research in the process of drug development and Union politics

Contract number:	HEALTH-F5-2007-201187
Project Type:	Specific Support Action (FP7)
EC contribution:	€ 317 964
Starting date:	1 April 2008
Duration:	24 months

Background

The basis of the START-UP project was the general intention to cover all the issues of 3Rs-bottlenecks in pharmaceutical research and development (R&D), as represented by the abbreviation, i.e., **S**cientific and **T**echnological issues in 3Rs **A**lternatives **R**esearch in **T**he process of drug development and **U**nion **P**olitics.

In order to have as much coverage as possible, the area was intensively analysed in expert meetings, attended predominantly by industry, but also by academia and regulatory authorities. Later on, in the 2nd year of the project, these closed expert meetings were extended to three open workshops on each of the 3Rs.

All in all, out of a total of 223 participants, there were 109 industrial experts (representing 42 companies) within the pharmaceutical industry or associated institutions, thereby reflecting in particular all aspects of "pharmaceutical life" in R&D. Started at a kick off meeting in Leverkusen at Bayer AG, the expert meetings in Madrid (Ministeria de Sanidad), Basle (Novartis Research Center), and Alicante (Pueblo Acantilado, at the biannual eSI meeting) were then followed by workshops on Refinement (Istituto di Sanita, Rome, IT), on Reduction (University of Innsbruck, AT), and on Replacement (Budapest, HU). These were collaborations of ecopa's National Consensus Platforms (NCPs) of Italy, Finland and Poland, respectively; Austria and the Netherlands, respectively; and Hungary and Germany. Academia was represented by 65 participants; regulatory authorities by 29; and animal welfare by 10. Also locally interested scientists actively participated.

The results were presented and found entrance into the discussions; the format varied, intentionally, from brainstorming sessions and working group-style to formal scientific presentation workshops or plenum style forums to enable free and interactive communication. All presentations and discussions are detailed in report form and are accompanied by an executive summary and a list of specific recommendations. The major outcome is present here as an overall executive summary, followed by the most prominent recommendations and a road map.

Some of the topics discussed might be subject to future projects within coming EU Framework Programmes.

Collation of 3R-topics in Pharmaceutical Research

Animal experiments are still needed and realistic progress is actually 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 works is, for the pharmaceutical industry, of greater 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). There should also be greater use of human cells, and more attention paid to 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., 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/exposure scenarios.

Important fields for further development are teratogenicity and embryotoxicity, as these tests are necessary for every newly developed drug coming on the market; for exploration of new opportunities for pharmacodynamics; and for better integration into single test programmes for pharmacokinetics, carcinogenesis, safety pharmacology and toxicology.

In test development, more focus should be placed on “risk assessment” than on “hazard assessment”.

Concepts of Cell System Improvements

These were high on the agenda. Stabilisation (e.g., by epigenetic modifications, miRNA interaction) of existing cell systems, and to use these for long-term testing has potential in 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.

Concepts of Data Sharing and Reporting of ''Negative'' Results

These aspects are important in gaining more basic information and reducing replication of experiments. They are of special importance in certain 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.

Aspects of Animal Husbandry and Best Practice for Animal Keeping

Emphasis was given to positive aspects such as better training of personnel and, in particular, of competent authorities, in relation to 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 Methodology

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, such as knock-out and transgenic animals, could help to make more appropriate use of animals as high target-specificity is involved. Also, transgenic cells, enzymes, and *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, micro-

biological high quality of animals, use of well-defined environmental conditions and techniques are crucial reduction parameters in this field.

Special Case of Vaccines Quality Control

As in the EU, authorities request that all vaccines lately must be tested, and as a consequence, high numbers of animals are consumed. 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 vaccines quality control, refinement strategies should be developed and implemented. Implementation of existing 3R-methods should be encouraged by improvement and global harmonisation of regulatory procedures. Also providing incentives to development and production is considered to be important. More attention should go to the neglected area of veterinary vaccines.

Specific ''High Burden'' Animal Disease Models

As animal pain models are not very predictive, well-controlled studies in man using microdosing were proposed in order to be able to score pain in a realistic way.

Cancer models are also a special target for further improvement, since by the development of biologics for this topic, the area is more 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 which usually exhibit the fully functioning pathways that are targeted. Also the importance of measuring *in vitro* specific biomarkers that can also be detected in the clinical situation came up.

Analysis of Union and 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 is seen as

a hindrance to appropriate research in alternative methods. Less bureaucracy, better integration of research teams, eventual leadership of 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 86/609 could help in this process.

In summary, the EU 7th RTD Framework Programme project START-UP has delivered a whole landscape of ideas and potential avenues for further research and development projects within the future EU Framework Programme 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. Also, these approaches, as laid out in more detail in the individual recommendations, have to be discussed with the experts involved, the Scientific Officers of the EU Commission, the European Parliament representatives as well as the industry and the interested public. With the pool of experts brought together under START-UP, the furthering of the attached Road Maps can be achieved.

Further workshops organised by the project partners involved should spread the message, in order to come up with solutions for some bottlenecks where solutions are not easy to come by.

Recommendations

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. Here the eight most important recommendations are summarised:

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 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 the animal model came up in the case of biopharmacuticals. The use of non-human primates (in a number of indicated cases), humanised models and transgenic animals seems relevant.
4. A lot of animals could be spared without loss of quality in the quality control of vaccines in Europe. Therefore it is 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 also 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: 3D-models, co-cultures, epigenetically stabilised cell lines, stem-cell derived specific cell types, etc.
7. Promotion of positive welfare of experimental animals, besides minimalisation of suffering, is seen as a refinement priority and should include active improvement of the degree of animal welfare in- and outside of experimental procedures, backed up by ethological studies on laboratory animals.
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.

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3 EU MEMBER STATE & INTERNATIONAL 3Rs INITIATIVES

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, this report includes for the first time a section devoted to updates from EU member state and international centres and initiatives who are working to advance the 3Rs and/or science of toxicology. Included in this edition are manuscripts of presentations delivered at the **AXLR8**-1 workshop. It is hoped that in future years, there can be increased EU national and international involvement in the **AXLR8** project.

CARDAM

Centre for Advanced R&D on Alternative Methods

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Background

CARDAM, the Centre for Advanced Research and Development on Alternative Methods, was founded by VITO in 2006 in order to promote and develop new alternative methods in response to the recent driving forces and needs for alternatives to animal testing. VITO is an independent research organization located in Flander's (Belgium) and accommodates more than 600 researchers. CARDAM contributes to VITO's strategy on sustainable development which is focused on the development and introduction of technological solutions accelerating the transition to a world reconciling human needs and the carrying capacity of the earth. VITO inspires, demonstrates and implements integrated solutions which contribute to smart processes and conserve ecosystems and protect human health.

For more than 20 years, VITO has performed research on environmental toxicology applying alternative methods. With the creation of CARDAM, these research efforts will be further developed into industrial applicable assays using high quality standards involving toxicogenomics, computational tools and state-of-the-art image analysis. CARDAM incorporates all in vitro toxicology services of VITO and operates as an independent business unit within the VITO structure. CARDAM's primarily objectives are in line with the 7th Amendment of the Cosmetic Directive, the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) programme, the EPAA (European Partnership on Alternatives to Animal Testing) organisation and the increasing awareness of researchers to change current safety testing strategies in accordance with the 3Rs (Replacement, Reduction, Refinement) principle. The R&D focus of CARDAM is towards

replacement and reduction of animal experimentation.

Mission

As a research organisation, CARDAM aims to become a European Industrial Centre of Excellence on alternative methods development by performing fundamental research as well as offering GLP compliant testing services and training on innovative and reliable alternative test methods for animal tests. These can be used individually or combined in test batteries, to help industry in selecting safer products for human and environment.

Strategy

In collaboration with industry, academia, and regulatory bodies, CARDAM focuses on four main activities:

- To perform research on alternative methods for animal testing with the aim to deliver industrial applicable methods in the fields of genotoxicity, eye and skin irritation, skin- and respiratory sensitisation, immunomodulation, reproductive toxicity with focus to developmental toxicity, developmental neurotoxicity and endocrine disruption.
- To develop and validate new promising methods towards an industrial applicable method according to internationally accepted processes as detailed by ECVAM (Hartung et al., 2004).
- To bring the validated methods in a GLP environment as a service to industry to meet the regulatory requirements for compound registration. CARDAM also aims to fine-tune these methods to screening purposes to help industry with selecting the most promising compounds (compound profiling) for final development.
- To train and educate scientists in the field of alternatives for animal testing.

Research Activities

The development and application of alternative test systems to evaluate the hazard of chemicals, has been stimulated and financially supported by VITO's strategic research programme, which sponsors several PhD projects in academia, and by the EU framework programmes. This allowed us to obtain scientific recognition and build research networks and partnerships in an international context. The focus was initially on environmental chemicals. Several alternative test systems, used for environmental toxicology research have been further developed and validated in the context of chemical safety testing. Recent developments combine *in vitro* cell culture technology with genomic analysis, complex data mining and biological pathway analysis.

The programme on emerging pollutants (endocrine disruptors, dioxin like compounds, non-dioxin like PCB's) has been supported by different EU Framework Programmes (MENDOS, DIFFERENCE,

ATHON and ReProTect) and has allowed to develop, implement and apply various cellular receptor transactivation tests (arylhydrocarbon receptor, estrogen receptor, androgen receptor). The assays were optimised or internationally validated (Freyberger et al., 2010; Schenk et al, 2010; Witters et al., 2010).

CARDAM is currently applying the newly developed zebra fish embryo test, an alternative for teratogenesis and for neurodevelopment (Selderslaghs et al., 2009; Selderslaghs et al., 2010) to detect developmental and behavioural effects of non-dioxin-like PCBs, for comparison to rat behavioural studies as were performed within EU-ATHON.

For detecting genotoxic compounds, the Vitotox® test was developed. This is a high throughput bacterial genotoxicity assay, which detects DNA damage caused by genotoxic compounds as light emission changes depending on SOS DNA repair. The Vitotox® test is now a licensed and trade marked product (Vanderlelie et al., 1997).

The asthma and allergy programme of VITO resulted in the VITOSENS® test which is an assay for the identification of skin sensitising low molecular weight chemicals. The assay is based on gene expression changes in human cord blood derived dendritic cells after chemical exposure (Hooyberghs et al., 2008). The VITOSENS® is patent pending. It is the first *in vitro* assay for sensitisation that has been developed making use of full genome arrays. The assay is ready for prevalidation. The programme on test development for sensitizers has been partly funded by EU Sens-it-iv programme. Test development for respiratory sensitizers is underway.

CARDAM will further focus its activities towards developing alternative tests for regulatory purposes which need to be performed under REACH for most of the compounds (Annex VII for > 1 tonne and Annex VIII for > 10 tonnes) and for regulatory tests which consume a high number of animals e.g., reproductive toxicology tests (Annex IX) (Schoeters, 2010). Research activities remain partly in-house but include also partnerships with academia, involvement through European Framework Programmes and active collaborations with industry. This collaboration ensures that applicable methods are being developed for regulatory testing, screening and selection of toxic compounds for development of safer pharmaceuticals, food ingredients, cosmetic ingredients and chemicals.

Further developments will include:

- Toxicogenomics which has already shown its usefulness in research for defining gene profiles and pathways relevant to the toxicities assessed. Cell-based techniques, toxicogenomics and proteomics will take the front-line at CARDAM in setting up *in vitro* screens and new test models to predict for a wide range of toxicities. Examples include the development of an *in vitro* potency assay for sensitising compounds, both skin and respiratory (Lambrechts et al., 2010; Verstraelen et al., 2009).

- Focus more on 3D cultures to have built in the test models cell-cell communications between different types of cells which may result in different outcomes compared to single cell cultures (e.g., 3D models for irritation, sensitisation, mutagenicity, etc.) (Kándárova et al., 2006).
- Focus more on the use of lower organisms to mimic more what happens in living organisms. Examples include a broader use of the zebra fish as test organism for other toxicities, the development of new applications of the Slug Mucosal Assay (InvertTox) such as "Stinging Itching Burning" (Adriaens et al., 2008).

Validation of Newly Developed Models

CARDAM collaborates with governmental organisations, the EPAA and industrial trade associations to develop and validate alternative models for regulatory requested animal tests.

CARDAM participated already in various international validation programmes. This allows us to get in touch with new upcoming tests. With the European Centre for Validation of Alternative Methods (ECVAM), CARDAM participated in the prevalidation of haemopoietic colony forming assays (Pessina et al., 2001), hER transformed HeLa-9903 assay for estrogenic activity, 3 fish cell lines for acute aquatic toxicity. Collaborators of CARDAM also participated in the pre-validation of the SkinEthic 3D-eye irritation model (Van Goethem et al., 2006) and in the slug mucosal irritation test (Adriaens et al., 2008). Currently, CARDAM is participating in an ECVAM co-ordinated, COLIPA-sponsored validation project on 3D-eye irritation cultures. CARDAM is also involved in the international validation of the OECD draft guideline, Fish Embryo Toxicity (FET), as replacement of adult fish acute toxicity (OECD TG203), which is co-ordinated by ECVAM and OECD.

Servicing Industry

- CARDAM combines the structure of a research organization with contract research services where it has to find the right balance between basic research and contract work. By conducting contract work, CARDAM aims to become financially more independent from VITO.
- A key factor for success to perform contract work for industry is GLP accreditation. CARDAM asked in 2008 for a GLP inspection, got its pre-inspection on 29 May 2008 and its final inspection on 28-29 January and 3 February 2009, with a certification in April 2009.
- Besides delivering toxicological and ecotoxicological services to the industry, CARDAM collaborates with industry to deliver more well-founded alternative tests suitable for their research. This also avoids duplication of work and efforts in both organisations in order to deliver well-balanced test methods. An example is to further develop and valorise the

BCOP assay allowing better prediction of eye irritation potential of test compounds based on an improved opacitometer (Van Goethem et al., 2010) which will be further improved at CARDAM.

Educate & Consult Scientists

To stimulate the implementation of alternatives, CARDAM will set up training sessions and workshops in the field of alter-

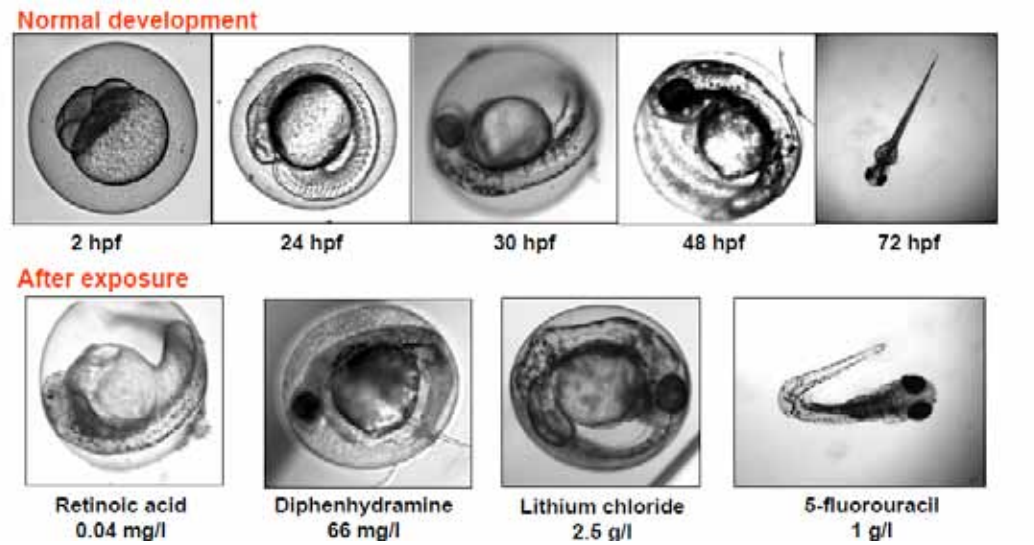
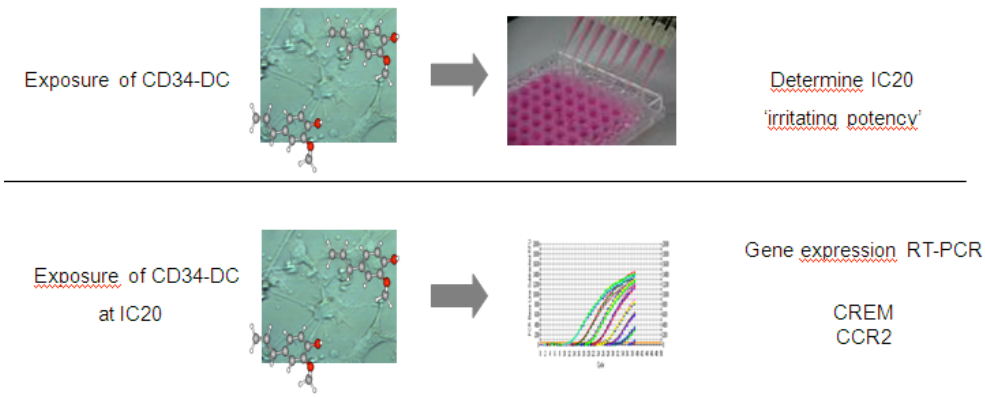


Figure 1. Embryos with malformations after exposure to a test compound (hours post fertilisation).

Figure 2. VITOSENS as a dichotomous classifier of (non)-sensitisers based on CD34+ stem cells obtained from human cord blood. IC20: the concentration which induces 20% inhibition of cell growth.



natives. A workshop was already held in 2009 for the Belgian Deontological committee reporting to the Minister of Health and Welfare.

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FICAM

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The Finnish Centre for Alternative Methods (FICAM) was established on 10 December 2008. FICAM is located in the Medical School at the University of Tampere. The main actions of FICAM are financed from the budget of the Ministry of Education. Additional research work is financed from various sources on project basis. FICAM focuses on development and validation of cell and tissue cultures, especially 3D-models to complement and/or to replace animal experiments. FICAM is the centre of expertise for alternative (replacement) methods in Finland. It shares information on alternative methods and implements alternative approaches in education and advanced training courses. FICAM also provides Good Laboratory Practice (GLP) and validation expertise on *in vitro* methods.

The Medical School of Tampere University has long experience in human cell and tissue research and the development of related technology. Thus, the majority of the test models under development in FICAM will use human tissues and cells. The human primary cells are isolated from the tissue material obtained from the Tissue Bank Finland (FinTiB) located in the Tampere University Hospital next to FICAM. Many of the tissue models use stem cell differentiated human cells. FICAM applies Good Clinical Practice (GCP) principles for human tissue material. The routine testing in FICAM is performed under GLP.

The test models developed and validated can be used in safety studies (e.g., pharmaceuticals, industrial chemicals, cosmetics) and efficacy testing (pharmaceuticals). The present test developments cover angiogenesis, neurotoxicity, several hormonal models, basic cytotoxicity and kinetic live cell imaging. Because of the expertise gained and of the application of GLP, FICAM is a competent reference laboratory, e.g., for the purposes of method validations for the European Centre for Validation of Alternative Methods.

3Rs Activities in Japan

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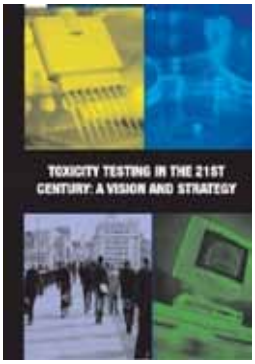
The Act on Welfare and Management of Animal revised in 2005. Since then, it has became widely accepted to use alternative methods to gain as much as possible and serve animals, proper animal due to reducing the number of animals that are available to users as possible shall be considered to use in the extent of the use of scientific purposes. Though Japan's 3Rs expansion is slower compared with western countries, it is making progress steadily.

This year, the Ministry of Health, Labour and Welfare (MHLW) created the Japanese Center for the Validation of Alternative Methods (JaCVAM) at the National Institute of Health Sciences (NIHS). JaCVAM has promoted the 3Rs in animal experiments for the safety evaluation of chemical substance in Japan and established guidelines for new alternative experimental methods through international collaboration for four years. Many Japanese colleagues have supported JaCVAM activities by performing validation studies, independent scientific peer reviews and by providing regulatory acceptance for new alternative experimental methods. Furthermore, we must plan to push forward with international harmonisation efforts in accordance with by both OECD activities and the International Cooperation on Alternative Test Methods (ICATM) framework, which was organised in April 2009.

On the other hand, Japan Health Sciences Foundation established the Centre for Accreditation of Laboratory Animal Care and Use in 2007. The centre aims to promote the optimum enforcement of scientific animal testing. The centre assesses and verifies institutes of animal experiments for their compliance with the "Basic Guidelines for Proper Conduct of Animal Testing and Related Activities in the Research Institutions under the Jurisdiction of the MHLW". The other ministries control animal experiments in universities and other institutes.

We think developments and continued activities of these Japanese centres will contribute much to achieve the 3Rs. We also expect these Japanese activities to contribute to International harmonisation in the 3Rs.

Toxicity Testing in the 21st Century



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The Vision & Approach

The United States National Research Council of the National Academies issued its report "Toxicity Testing in the 21st Century: A Vision and a Strategy" in 2007, providing a roadmap for a transition in toxicity testing. The current system, based largely on animal tests and the identification of phenotypic changes, is widely recognised as being too costly, inefficient, and wasteful of animals who, in any case, may respond differently to toxicant challenges than humans (NRC, 2007).

At its core, the report proposed that a new testing strategy be designed that relies upon the best science, seeking a molecular and cellular mechanistic understanding of toxicant mode of action. Toxicity pathways, normal biological pathways involved in toxicant responses, were highlighted as the key building blocks of this new approach. An initial phase in the development of the new testing strategy involves a description of the toxicity pathways. Once these building blocks are described, the report proposes taking a systems biology approach to integrating toxicity pathway responses into a mode of action framework based on dose response. At a sub-threshold dose, a pathway may begin to be disrupted by a toxicant exposure, but continue to function. Above some threshold, the function of a pathway may be compromised. The characterisation of dose-dependent transitions in pathways, and the integration of responses across pathways will be used to identify mode of action and adverse effects. The new knowledge gained contributes both to improved toxicity testing and to the fundamental molecular elucidation of human disease.

Implementation

Three years after its release, now is a good time to review the original proposal for implementation in the report, and progress to date. The report indicated that implementation was best served by the following:

"A long-term, large-scale concerted effort ..... to bring the new toxicity testing paradigm to fruition... many years and hundreds of millions of dollars....noticeable changes in toxicity-testing practices within 10 years... within 20 years, testing approaches will more closely reflect the proposed vision than current approaches.... a research institute that fosters multidisciplinary research intramurally and extramurally... funded primarily by the federal government" (NRC, 2007).

Significant progress has been made, with a high profile publication initiating a process of federal agency commitment to collaboration and sharing of resources (Collins et al., 2008). The US Environmental Protection Agency (EPA) National Center for Computational Toxicology has also made rapid progress in developing its approach to new in *vitro* tests for hazard identification, as illustrated in a recent publication (Judson et al., 2010). The launching of Tox21 brings together EPA, the National Institute of Environmental Health Sciences, the National Institutes of Health Chemical Genomics Center, and the Food and Drug Administration, in a cooperative arrangement to further the technology with concerted effort and investment. While these federal agencies are spending millions of dollars per year, the investment is less than the report envisioned as being required. In addition, the lack of a single institution directing the effort, and the failure to significantly engage national and international extramural partners remain limitations on the current approach. Overall, however, the track record on implementation is substantial, positive, and growing.

Redefining "Adversity"

One component to examine in the implementation of the paradigm shift in toxicity

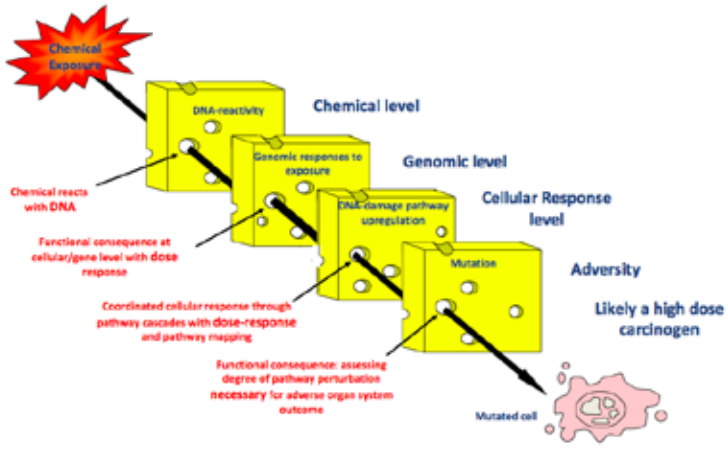

Figure 1. Defining adversity from panels of assays (reprinted with permission from Boekelkeide and Andersen, 2010).

testing is the way in which adverse effects are understood. Adverse effects are currently defined by referring to endpoints related to whole animals. A commonly used definition of an adverse effect is "A biochemical, morphological or physiological change (in response to a stimulus) that either singly or in combination adversely affects the performance of the whole organism or reduces the organism's ability to respond to an additional environmental challenge" (Lewis et al., 2002).

Defining adversity by reference to endpoints at the level of the whole organism is incompatible with a systems biology based toxicity testing approach that relies upon an understanding of toxicant-induced modes of action at the molecular level. Therefore, a new framework for defining adverse effects has been proposed that accommodates the emerging paradigm of toxicity testing in the 21st century (Boekelheide and Campion, 2010). The new framework is based upon the experience of accident investigators, and identifies latent and active failures within categories of the testing system database (Figure 1).

Summary

Three years after the publication of the NRC report, the new approach to toxicity testing has garnered significant attention and support. Ongoing efforts within several federal agencies, notably ToxCast™ and Tox21, continue to grow. While renewed attention to fundamental issues is required (such as whether the new toxicity testing system is designed for screening and prioritisation or as a stand-alone system), there has been significant progress in the design and development of assays and approaches. Continued basic and applied research will be needed to bring about full implementation.

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ToxCast & Tox21

Providing high-throughput decision support tools for chemical risk management

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Overview

As noted by the United States National Research Council (NRC, 2007) in its vision for a new paradigm in toxicity testing, 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, their remains large uncertainties in extrapolating data across dose, life stage and species. The magnitude of the chemical information gap was quantified by Judson et al., (2009), who pointed out that the majority of chemicals of concern to the US Environmental Protection Agency (EPA) have very limited toxicity studies available in the public domain. The EPA responded to the vision of for a transformation in toxicology proposed by the NRC (2007) by developing a strategic framework upon which the agency could comprehensively move forward to incorporate the new scientific paradigm into future toxicity testing and risk assessment practices (EPA, 2009; Kramer et al., 2010). This Strategic Plan recognises that the challenges EPA and sister regulatory agencies face in implementing such a transformation are significant, as the new toxicity testing paradigm will markedly change the nature of the current methods, models, and data that inform hazard identification, dose-response estimation, exposure assessment, and risk characterisation. The EPA Strategy is centred on three interrelated issues: (1) the use of toxicity pathways information in screening and prioritisation of chemicals for further testing; (2) the use of toxicity pathways information in risk assessment; and (3) organisational transition. The last element explicitly recognises that regulatory offices within EPA will need to be actively involved in

overseeing the significant transition to this new paradigm and the translation of the attendant data for regulatory application.

Research to address the first issue is building on the efforts of EPA's ToxCast™ programme in identifying and developing simple, reliable screening models to predict chemical hazard (Dix et al., 2007). The second effort will seek to apply the toxicity pathways concept in a systems biology approach, i.e., to better delineate the molecular and cellular changes that perturb normal homeostatic mechanisms towards a given toxicity pathway or set of toxicity pathways. This information should reduce the uncertainty currently associated with dose-response models by increasing their biological plausibility. The efforts of EPA to implement this strategy are covered in Kavlock and Dix (2010).

A fundamental key to the success of any new paradigm in toxicity testing is that of chemo-informatics (Richard, 2006). Toxicity databases must be established on standardised schema, developed in conjunction with subject matter experts for specific areas of toxicology and populated with extensive data extractions are absolutely essential to support the maturation of predictive toxicology. The effective capture and representation of legacy data (e.g., Martin et al., 2009a,b; Knudsen et al., 2009) illustrate the utility of building the informatic infrastructure. Coupled with this is the need to carefully curate the information on the chemicals, such as demonstrated by the DSSTox project (*EPA.gov/NCCT/dsstox/index.html*). Providing a downloadable, structure-searchable, standardised structure files associated with toxicity data ensures that structural analogues can be identified and that divergent data sets, such as being generated in high throughput screening, can be compared. Combining HTS results with physical chemical properties creates the opportunity to create structure-bioactivity relationship (SBARs) that should be more robust in generating predictions, as they incorporate aspects of both chemical and biological space.

Having scientifically accepted predictive tools will enable the more efficient and effective characterisation of chemical risk and hazard, and lead to a more rational use of animals in research as testing is directed to the highest priority chemicals. Historically, most attempts to meet the need for predictive tools have focused on the development of quantitative structure-activity relationship (QSAR) models. Although QSAR models have proven useful for predicting some mechanisms within relatively well-defined chemical classes, they often do not perform well when screening broadly diverse categories of chemicals because their training sets do not adequately cover the relevant chemical space. Therefore, other approaches are needed, and the experience of the pharmaceutical industry in the use of state-of-the-art high-throughput screening assays (HTS), toxicogenomics, and computational chemistry tools for the discovery of new drugs might provide a solution. HTS refers to a system that rapidly and efficiently tests large numbers (i.e., thousands) of chemicals for bioactivity, typically utilising robotics and automation applied to

biochemical and cellular assays (Inglese et al., 2006). Exploiting recent advances in HTS and toxicogenomics, EPA has launched a research programme called ToxCast™ to develop methods for prioritising chemicals for further screening and testing (Dix et al., 2007).

The pharmaceutical industry, in its efforts to develop more efficient methods for drug discovery, is responsible for many of the new tools available to predictive toxicology. Information on the success of these new tools is limited due to confidential business practices. However, the limited information that is available suggests that these tools should be applied to detecting the health hazards associated with environmental chemicals (reviewed in Houck and Kavlock, 2007). HTS systems have been used to evaluate the modulation of drug metabolising enzymes, genotoxicity, ion channels, receptor binding and activation, biochemical targets such s GPCRs, kinases, proteases, phosphates, phosphodiesterases and other protein families, complex cellular assays involving high content imaging of fluorescent probes and model organisms such as *Drosphila, C. Elegans, and D. rerio*.

ToxCast™ is designed to test the hypothesis that multi-dimensional evaluation of chemical properties and effects across a broad spectrum of information domains (e.g., molecular, cellular, and organ responses). It is 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. With a commitment to transparency and public release of all data, it is most strategic and coordinated 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 physical-chemical properties, predicted biological activities from structure-activity models, biochemical properties from HTS assays, cell-based phenotypic assays, genomic analyses of cells *in vitro*, and responses in non-mammalian model organisms. Phase 1 of ToxCast™ involved the evaluation of 309 unique chemicals against a battery of 467 *in vitro* assays from different technology platforms, and the results were recently published (Judson, et al., 2010). Phase 1 demonstrated that a broad spectrum of chemical activity at the molecular and pathway levels, with chemicals interacting with mean 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 show widely varying promiscuity across pathways, from no activity to activity against dozens of pathways. Interestingly, there was a statistically significant inverse association between the number of pathways perturbed by a chemical at low *in vitro* concentrations and the lowest *in vivo* dose at which a chemical

first causes toxicity, suggesting that even a simple measure like promiscuity in *in vitro* assays can be used to prioritise chemicals for *in vivo* evaluation.

The ToxCast™ chemicals were largely derived from a list of food use pesticides, and hence are generally regarded as non-genotoxic chemicals. However, twenty-one of the ToxCast™ 309 chemicals were shown to induce liver tumours in chronically exposure rats. This bioactivity signature suggests that is a chemical interacts with the peroxisome proliferating activated receptor gamma pathway (PPARγ) and one of more of the following pathways: PPARα activation, cytokine CCL2 up-regulation (CCL2), androgen antagonism (AA), or oxidative stress (OS), 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 by the US National Toxicology Program (Michael De Vito, personal communication).

ToxCast™ is now entering Phase II, which will examine the effects of an additional 700 chemicals against a similar range of assays. 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 chemicals, and chemicals used as food additives, which also have a rich toxicological database. The expanded diversity of chemicals in this phase was selected to more adequately the types of chemical structures that would be the target of ToxCast™ should it be implemented to screen and prioritise chemicals for further testing.

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), would be grouped together into 'domains' 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 an interval value [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 up endocrine related activity, domains included in estrogen, androgen, thyroid and other nuclear receptors interactions all derived from *in vitro* bioassays, physical chemical properties such as log P and total polar surface area (TPSA) derived from computational models, and disease pathway domains obtained from mapping the *in vitro* assays against several reorientations 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 sources. 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 piece (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 (Figure 1).

Alternative implementations for hypothetical prioritisation tasks within this framework could include, for example, additional domains have been included to represent knowledge from *in vivo* study results, exposure estimates or other additional chemical descriptors such as a collection of QSAR models or a specialised prioritisation task such as developmental toxicity, wherein a targeted set of chemical properties related to placental transport would be especially important. While all domains are currently given equal weighting, expert judgment could be used to adjust various domains to carry more or less weight in the overall ToxScore™ calculation (in essence, given certain slices larger or smaller radians in the ToxPi visualisation.

ToxCast™ is part of a much broader US Government research collaboration called Tox21. Prior to the NRC report in 2007 these efforts, which consisted of the National Toxicology Programme, through its Roadmap (NTP, 2004), the NIH Chemical

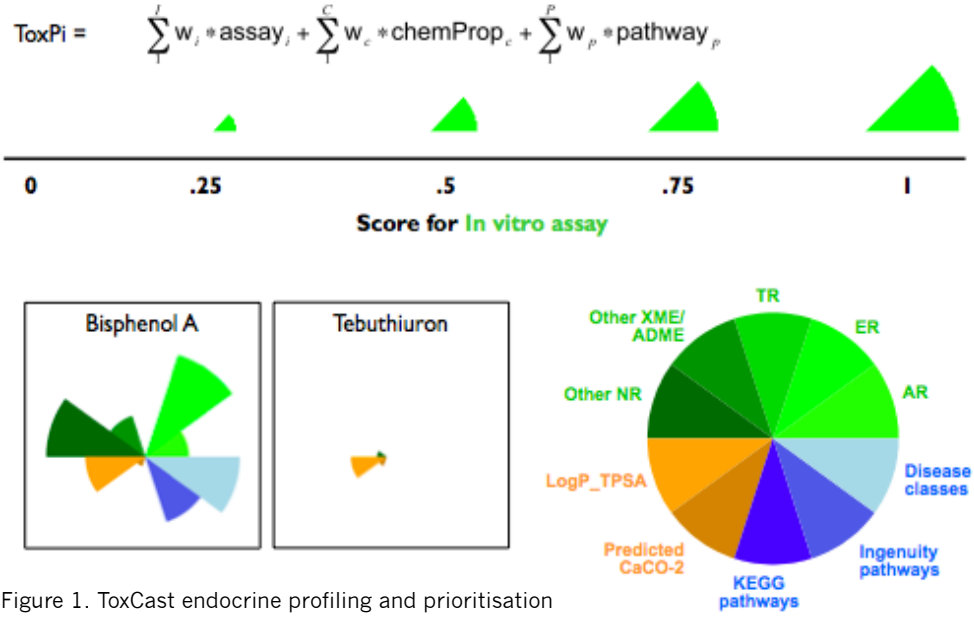

Figure 1. ToxCast endocrine profiling and prioritisation of environmental chemicals, visualised using ToxPi.

Genomics Center through its Molecular Libraries Initiative (*NCGC.nih/gov*) and the EPA through it's ToxScore™ programme and Strategic Plan for the Evaluating the Toxicity of Chemicals (EPA, 2009) were only loosely coordinated. Spurred on by the NRC report, those three government agencies entered into a Memorandum of Understanding in February 2007 (Collins et al., 2008) to bring their expertise and complementary capabilities to bear on transforming the conduct of toxicological evaluations. A central aspect of the Tox21 consortium is the ultra high throughput capabilities of the NGCC, which can profile the effects of thousands of chemicals against an *in vitro* assay in single. Four working groups operate within Tox21—chemical selection, assay selection, informatics and targeted testing (Kavlock, Austin and Tice, 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 occur in the literature (Xia et al., 2009). Currently the consortium is developing a much library of 10,000 chemicals, contributed equally by the three members, with screening scheduled to start in late 2010. Approximately one HTS per week will be conducted on this library over a likely two-year period. The Food and Drug Administration joined Tox21 in 2010, and discussions are under way with the European Commission Joint Research Centre in Ispra, Italy to expand this to an international collaboration.

DISCLAIMER: The United States Environmental Protection Agency through its Office of Research and Development reviewed and approved this publication. However, it may not necessarily reflect official Agency policy and reference to commercial products or services does not constitute endorsement.

Postscript

On 6 October 2010, the European Commission announced the establishment of a transatlantic partnership between the Joint Research Centre and the US EPA to exchange research materials and results useful for the development of integrated methods for predicting chemical toxicity. One of the aims is to use the JRC as a hub for networking among research groups and organisations in Europe who want to join forces in this initiative, working in partnership with projects such as **AXLR8**.

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OECD Activities

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The Organisation for Economic Co-operation and Development (OECD) has a long history of test method validation and development for regulatory purposes worldwide and many of the ongoing FP-funded projects aim at developing alternative test methods that may replace *in vivo* test methods in the OECD Guidelines for the Testing of Chemicals in the future. The OECD is very active in the area of developing alternative Test Guidelines and applying the 3R-principles, and non-testing methods (QSAR, read-across, chemical categories, etc.) are high on the OECD agenda. The work programme on chemicals now includes work on new approaches, such as molecular and high-throughput screening based on the US ToxCast™ programme, and the first workshop on the development of integrated approaches to testing and assessment was held in the US in 2008 (OECD, 2008). A focus session was recently held by the OECD Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology to discuss current and forthcoming approaches to chemical safety and animal welfare (OECD, 2010). It should also be emphasised that it is important to deepen communications between the research society and the regulatory community to facilitate development of alternative test methods that both meet the requirements set by the regulatory community as well as represent the latest and most modern techniques available.

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4 DISCUSSION & RECOMMENDATIONS

The contents of this chapter reflect the views of **AXLR8** Scientific Panel members articulated at the time of the **AXLR8-1** workshop in Potsdam, and also points arising from the **AXLR8** Info Forum panel discussion in Linz. The discussion in both events was lively and productive. Comments and recommendations of a general nature are outlined below, whereas feedback specific to individual FP6/7 projects has been collated and provided to project coordinators on a private and individual basis.

Reflections on European 3Rs Research

The **AXLR8** Scientific Panel concluded that substantial progress is being made in Europe in the development of 3Rs test methods and integrated testing strategies, but that as a general point, progress reports from FP-funded projects could in future be improved by indicating areas in which research extends beyond the state-of-the-art. Overarching points arising from Panel's discussion include the following:

Regulatory Requirements

- The Scientific Panel acknowledged that in the EU, the development of *in vitro* toxicity tests for regulatory purposes has thus far been mainly "policy-driven", i.e., by legislation such as the 7th Amendment to the Cosmetics Directive and the REACH chemicals regulation, which mandate swift action/deadlines for replacement of animal use in toxicity testing. In some instances, specific toxicological endpoints are named in the legislation, which has inspired a series of endpoint-oriented, "bottom-up" research initiatives. This approach has yielded a number of potentially useful tools; however, the lack of an overarching and unifying "top-down" strategy has been cited as a limitation to be corrected in future.

- Where new methods are developed, these should be based on human-source material rather than cells and tissues obtained from rodents or other animal species.
- To reach regulatory acceptance, new *in vitro* test should predict effects in humans under realistic conditions of exposure, rather than effects observed at very high concentrations. Mathematical (e.g., physiologically-based pharmacokinetic or PBPK) models will be needed to allow extrapolation from effects observed in simple *in vitro* systems to the more complex *in vivo* situation.
- When new *in vitro* methods are developed for toxicity testing, it should be defined up-front if the new tests will be used to meet regulatory requirements or to support in-house safety decisions, e.g., on consumer products.
- To meet the challenges of the 21st century, we do not only need a change of paradigm in the science of toxicology but also in regulatory hazard and risk assessment procedures (i.e., "risk assessment 2.0").

Cell Culture Standardisation

- There is a need to standardise cell sources and culturing protocols between laboratories that intend to generate data using the same test system. For instance, assuming that the nominal cell phenotype can be obtained, instead of trying to achieve perfect reproducibility, a suitable experimental design (e.g., normalised potency results with respect to reference chemicals) will allow compensating for slight differences in cell models, which would not fundamentally affect test performance.
- In relation to the development and optimisation of culturing protocols for stem cell-derived models for *in vitro* testing, it would be useful to have more specific descriptions of the characteristics desired for a specific model. Ultimately the aim would be to produce a stem cell-derived model that is fit-for-purpose, rather than being "perfect" in every aspect.
- The Panel also noted that laboratories in the EU are increasingly investing in modestly-sized assay automation systems, and suggested that the topic of high throughput automation and its role in EU-funded research projects merits a focused, stand-alone discussion. Through its HTS facility in Ispra, the JRC can help to facilitate the networking of academic/research assay-automation laboratories to encourage the exchange of experiences and know-how, and to consolidate testing capacities.

Metabolising Systems & Toxicokinetics

- The Scientific Panel noted that a variety of *in vitro* approaches to address the issues of metabolic biotransformation and metabolism-mediated toxicity have been developed in a number of FP-funded projects. It was also noted that a "virtual liver" project called HepatoSys (*http://www.hepatosys.de/en*) has

been awarded 80-90 million € in funding over ten years by the German Ministry for Education and Research. 3Rs projects in the EU and internationally are encouraged to actively cooperate with this and other relevant initiatives. Closer coordination and pooling of efforts should avoid duplication and accelerate progress.

- Rather than devoting more time and resources toward the development or improvement of human metabolising liver cell systems at the level of individual projects, preference should be given to the use of such well-characterised models as the HepRG cell line.
- The metabolic competence of hepatocyte models remains a source of discussion and speculation. Efforts should be made to quantify the activity of metabolising enzymes to eliminate doubts on the ability of a liver cell model to capture metabolism-mediated hepatotoxicty.
- PBPK models should be developed and incorporated in holistic systems to assist in the realistic estimation of target tissue concentrations.

Multi-Component Test Systems

- A number of projects are aimed at developing multi-component test systems based on a combination of multiple readouts. Such multi-parametric systems are challenging with respect to the mathematical approaches that should be employed to build prediction models, the generation of high-quality data on a sufficient number of chemicals to build a reliable model, and the approach taken to their validation.
- Similarly, a number of projects are using 'omics combined with *in vitro* cell models, both as the main output of the test system as well as for input into a prediction algorithm/model. These technologies offer considerable promise for elucidating the mechanisms of toxicity and modes-of-action. Progress could be enhanced by more inter-project and international discussion and collaboration.

Systems Biology & 'omics

- The Scientific Panel considered that the application of systems biology is promising for extrapolating data to different combinations of variables using mathematical modelling. Identifying gaps or missing information will add to the strength of modelling. Closer communication between biologists and modellers is recommended.
- Further development of systems biology-based mathematical models is encouraged, as well as experimental approaches for verification. For this purpose, more attention could be paid to the identification of translational biomarkers (e.g., biomolecular or morphological markers of clinical pathology) that are relevant and informative in an *in vitro* context.
- Although 'omics approaches are increasing in popularity, there is significant promise in novel functional bioassays. Such assays are particularly relevant in cases where an overly

reductionist approach will lose key pathways (e.g., neural networks embody pathways that depend on network function).
- Systems biology approaches to toxicology require "top-down" conceptual thinking rather than the traditional "bottom-up" approach to develop *in vitro* toxicity tests.
- There is a need for proof-of-principle studies demonstrating the added value of a systems biology approach to safety testing.

Selection of Reference Substances & Prevalidation

- The Scientific Panel observed that a number of EU projects have invested significant resources in the selection of reference compounds for the characterisation of test systems. This has led to the collection of significant quantities of chemical hazard reference data by multiple projects. To avoid unnecessary duplication of effort in future, a centralised repository/database of reference compounds should be created (e.g., at the JRC). Within the scope of this activity, the Panel highlighted the importance of gathering available human data in relation both to exposure and to intrinsic properties/hazards (e.g., from clinical drug trials, epidemiology, poison control centres, etc.).
- It was also noted that the development of test systems that are meeting "ECVAM's criteria to enter prevalidation" has been cited as a deliverable in several FP6/7 projects. However, the interpretation of these criteria and the term "prevalidation" are not applied in a stringent manner. The value of conducting prevalidation studies of candidate test systems within the life of an FP6/7 project and independent from ECVAM was also challenged. Although feasibility- and demonstration-type activities might be useful to show the potential of a test system, in some instances significant effort and project resources have been invested in formal prevalidation work on test systems, which in the opinion of the Scientific Panel may not warrant such efforts.

EU Approach to 3Rs Funding & Method Development

- The Scientific Panel appreciated that the EU approach to 3Rs development and research funding is closely linked to legislative mandates (e.g., cosmetics and chemicals), which has led to a focus on "apical" endpoints such as acute and reproductive toxicity, and the development of alternative methods and strategies aimed at replacing animal use on an endpoint-by-endpoint basis.
- There are many common elements that need to be addressed in many projects, e.g., metabolism, genetic variability, embryonic stem cell differentiation protocols, and these should not be addressed in depth in every project.
- During the **AXLR8** Info Forum it was noted that although the European "integrated project" model on the one hand promotes useful interdisciplin-

ary collaboration. However, "letting many flowers blossom" in the absence of a central strategy was cited as a substantial drawback. The division of resources in too many different directions, and too many loosely organised participants, can lead to inefficiencies and the repetition of mistakes. In contrast, a limited number of scientists working on a focused question can arrive at a tangible deliverable with relatively greater ease and efficiency.

- The lack of continuity and follow-up at the end of most EU projects was also cited as an area for improvement.
- It was suggested that projects funded under the European Commission/COLIPA joint research initiative[3] on repeated dose toxicity, as well as nanotoxicology- and ecotoxicology-related 3Rs projects, could be brought within the umbrella of the **AXLR8** coordination project.
- Finally, to answer the question whether the EU needs its own vision/strategy for "21st century toxicology", it was the general view of panel members at the **AXLR8** Info Forum that enhanced transatlantic coordination and collaboration are what are really needed.

[3] European Commission (2009). The European Commission and the cosmetic industry match research funds to develop alternative solutions to animal testing (news alert). Website *http://ec.europa.eu/research/index.cfm?pg=newsalert&lg=en&year=2009&na=na-310809*

Reflections on International Paradigms & Initiatives

Perspectives of AXLR8 Scientific Panel Members

- The central concept underlying the vision of "21st century toxicology" is that adverse health effects can be described by toxicological pathways that link together a chain of key biological events, from the first interaction of an exogenous chemical with an endogenous biomolecule, to the presentation of pathology and related symptoms. The definition, identification and categorization of these pathways are critical first steps in realizing the vision. Within the **AXLR8** project, thought should be given to organize a workshop to tackle this issue. The JRC might be in a position to support such an initiative.
- The US NRC (2007) vision of developing a "top-down" approach that will provide a new paradigm for risk assessment will take 10 to 15 years to develop and implement—or even longer, if the current fragmented approach to supporting alternatives development and implementation continues. This vision is not only a shift in paradigm—theoretical biology versus conventional *in vitro* biology—but also a shift in technology to high-throughput systems (HTS). Most of the HTS capacity in the US is delivered by two test robots at the National Institutes of Health Chemical Genomics Centre. The creation of an equivalent HTS infrastructure in Eu-

rope would be a costly but worthwhile investment.

- A paradigm shift away from focusing on morphological endpoints to molecular biology endpoints means that changes in signalling pathways will have an impact on phenotype. However, the basic biology/cellular pathology behind this concept is very complex and influenced by factors that may not be fully understood today. Only better understanding of these processes will enable reliance with confidence on pathway-based *in vitro* methods.
- The long-term vision of the US NRC is viewed by some as being incompatible with the more near-term mandates imposed by European legislation. However, for priority-setting purposes, a stepwise/phased-in approach would be very useful. For example, HTS methods under development by the US EPA ToxCast™ programme deliver mechanistic biological data on a large number of substances very quickly, which can be examined alongside exposure data in sorting and prioritising tens of thousands of existing substances for in-depth assessment. The interpretation of HTS data does, of course, raise issues; however, uncertainties seem not to be any greater than those associated with animal tests.
- Given the substantial and increasing investment in 3Rs and molecular toxicology research, both within and outside the EU, it is important that the coordination project **AXLR8** has been established to stimulate effective, real-time dialogue, information exchange, and problem-solving.

Perspectives of Participants in the AXLR8 Info Forum

- In Phase 2 of ToxCast™, investigators with novel *in vitro* systems are welcomed, because the complexity of real tissues is desired at this juncture.
- Tox21 partners each play a unique role, including now the European Commission/JRC, which has just signed a collaborative agreement with the US EPA National Center for Computational Toxicology.

Laying the Groundwork for a Pathway-Based Paradigm in Europe

- The **AXLR8** Scientific Panel concluded that the vision and strategy proposed by the US NRC (2007) represents an appropriate basis for a new approach to chemical toxicity testing and risk assessment. The Panel further noticed that, in addition to the recommendations listed above:
  - The rapid emergence of HTS/high-content test systems can best be exploited in the near-term in a hazard-profiling context, helping to uncover toxicity pathways and elucidate chemical structure/biological activity relationships. Only when a sufficiently developed systems toxicology framework has been developed will it be possible to build hypothesis-driven test systems with acceptable performance.
  - If *in vitro* models are to be used in

pathway-based toxicity profiling, developers should make an effort to identify the pathway(s) of a model rather than describing it in terms of a specific *in vivo* endpoint. This will facilitate the use of *in vitro* models within a new classification system based on defined pathways.

- While pathway-based *in vitro* models are being developed and verified, they should be introduced as early as possible into the regulatory pipeline, e.g., REACH provides opportunities to introduce emerging test systems and concepts to provide safety information.
- To successfully meet the challenges foreseen in the development of a pathway-based toxicology, a framework of transatlantic/pacific coordination is essential and should be given high priority by funding programmes in the EU, Japan and the United States.
- At EU level, efforts should be made to better integrate 3Rs research funding among the health and environment Framework Programme themes, as well as among multidisciplinary projects, e.g., regenerative medicine and disease pathways.

5 THE WAY FORWARD

Substantial progress is being made in Europe in the development, validation and regulatory acceptance of 3Rs methods.

Internationally recognised success stories in the areas of skin and eye irritation, skin absorption, phototoxicity and pyrogenicity would not exist without the long-term commitment and investment by the EU in research to advance the scientific basis of toxicity testing for the common goals of improved human safety and reduced animal use. Progress in several additional human health endpoint areas is foreseen as a product of research funded through the EU FP6/7 Health programme and related initiatives.

New technologies are emerging and creating a range of exciting opportunities and approaches. For the first time it is possible to envision a completely new concept for chemical/small molecule risk assessment, in which adverse health effects can be described by "toxicity pathways" that link together a chain of key biological events, from the first interaction of an exogenous substance with endogenous biomolecules, to the generation of pathology and related symptoms. This is the basis of the vision of "21st century toxicology" articulated by the US National Research Council[1] and others[2,3]. This vision has been endorsed by the **AXLR8** Scientific Panel as a science driven basis for chemical

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[1] NRC (2007). *Toxicity Testing in the 21st Century: A Vision and A Strategy.* Washington, DC: National Academies Press.

[2] Berg N, De Wever B, Fuchs HF, et al. (2010). Toxicology in the 21st Century—working our way towards a visionary reality. Willemstad, NL: *In Vitro* Testing Industrial Platform. Website *http://www.ivtip.org/images/IVTIP_publication-final.pdf.*

[3] European Partnership on Alternative Approaches for Animal Testing (2008). *New Perspectives on Safety: Workshop Report*. Brussels: EPAA.

toxicity testing and risk assessment.

Elements of the NRC vision are already present in some research supported by the European Commission and industry (Figure 1). These common "building blocks" include the pursuit of a molecular and cellular mechanistic understanding of the mode of action of chemicals, the development of metabolising *in vitro* systems to study chemical biotransformation, the advancement of systems biology and bioinformatics, and the refinement of physiologically-based pharmacokinetic modelling to enable meaningful *in vitro-in vivo* extrapolation. However, so far a clear strategy towards a common goal has been lacking. The individual building blocks need more cohesion and coordination, both on European and international levels. In the context of near- and longer-term legislative and scientific priorities, the development of a practical roadmap, which integrates new and existing European and global research programmes and defines knowledge gaps and future research needs and priorities, is the central challenge for the **AXLR8** project.

In the short-term, research should give the following aspects priority:

- Definition, identification and categorisation of key biological pathways in human toxicology.
- Development of an ontology and related taxonomy and vocabulary related to toxicity pathways and adverse effects.
- 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 PBPK/multi-scale modelling and other bioinformatics tools to interpret the substantial amount of new data that will be produced under the pathway concept.
- Proof-of-concept demonstration studies using known toxicity pathways and clinical symptoms/biomarkers to illustrate the concept.

Funding schemes should emphasise "value-added" collaborations among established research teams in key areas (i.e., the Tox21 initiative) to create synergies without duplication. For instance, there may be scope for promising *in vitro* methods emerging from FP6/7 projects. Targeted, multidisciplinary partnerships should also be encouraged, given that a solution for truly animal-free, predictive safety assessment needs the mobilisation of the best scientists in their fields, many of whom would not traditionally apply their work to toxicology.

As an encouraging start in Europe, an HTS facility has been established at the JRC Institute for Health and Consumer Protection in Italy to support more rapid and comprehensive assessment and validation of promising *in vitro* assays, and to generate high quality datasets to support the development of integrated strategies for predictive toxicology. The current capacity at the JRC for HTS and HCA studies is involving hundreds of

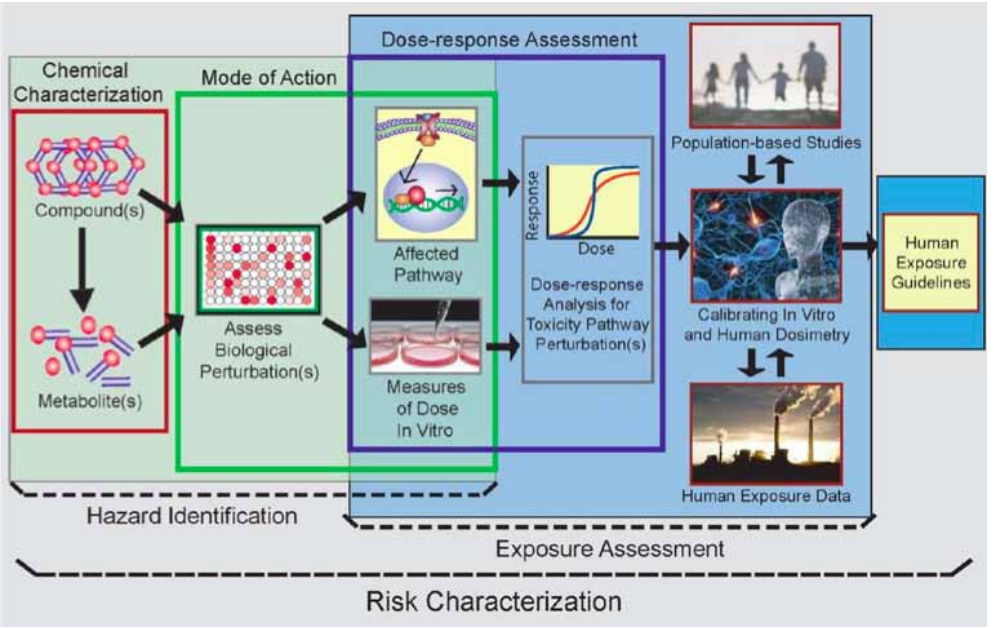

Figure 1. Risk/safety assessment following *Toxicity Testing in the 21st Century*[1]. The mapping is done against the four key components of the contemporary risk assessment paradigm: hazard identification, dose-response assessment, exposure assessment, and the overall process of risk characterisation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

reference chemicals in 96- and 384-well qHTS format. It is the intention of this facility to understand and show how HTS can be used in formal validation studies coordinated by ECVAM of other partners in the International Cooperation on Alternative Test Methods (ICATM). Within the collaboration between the Tox21 initiative and ICATM (ICCVAM-ECVAM in this case), it is planned to demonstrate the automation and scaling up of an ER-binding luminescence reporter assay within an international validation study. This collaboration model could pave the way for initial automation and evaluation at the JRC of candidate assays coming from research projects, followed by transfer and scaling up to 1536-well format at NCGC for testing the Tox21 10,000 chemical library.

A truly integrated multidisciplinary and multinational approach, in which scientists and regulators engage jointly towards a common goal would provide an exciting and unprecedented platform for exploring future approaches to safety assessment without animals. Between 1985 and 2005, the world's biomedical research establishment launched the first "big biology" pro-

[4] Krewski D, Andersen ME (2010). New directions in toxicity testing. *Ann. Rev. Public Health.* [In press.]

ject—the Human Genome Project. Today we are at a new biological milestone, where we could—with sufficient international and political support and the effective allocation of around 200 million € per year—produce the means and the technology to test for and assess the human and environmental risk of tens of thousands of chemicals per year without using animals. The challenge is whether we will achieve this goal in 30 to 50 years, or in 10 to 15 years.

It would be the most encouraging signal if high-level bilateral agreements on science and technology (e.g., under the Transatlantic Economic Council) would initiate a co-operation to meet the challenges of the "21st century toxicology" approach. This initiative has great potential for joint funding initiatives, and as illustrated above, there is plenty of work for everyone.

At the same time it is important not to over-promise and fail to deliver. The approach proposed will require a long-term investment before its full potential can be realised, i.e. near or total replacement of animal use. However, with each key milestone reached it should be possible to integrate new advances into regulatory decision-making in a practical way, thereby addressing EU legislative and public policy mandates. For instance, it has long been the intent of US regulators to apply the ToxCast™ *in vitro* test battery initially as a tool for screening large inventories of industrial chemicals and prioritising substances for further assessment. Quite unexpectedly, in 2010 during the devastating oil leak in the Gulf of Mexico, the ToxCast™ platform was successfully used to provide a solution for an urgent regulatory need, the rapid toxicity assessment of chemical dispersants[5], a task that due to time constraints could not be performed using conventional animal tests.

Although there is always a risk when trying to prove and implement novel scientific concepts in the life sciences, a "systems" approach to human biology that relies on mechanisms and pathways could hold great value for society, contributing both to improved toxicity testing and to the fundamental molecular elucidation of human disease. This will require strong and effective coordination—a service that the **AXLR8** project stands ready to provide.

[5] EPA (2010). Oil Spill Dispersant Screening Results. Website *http://www.epa.gov/ncct/download_files/factsheets/Technical%20Fact%20Sheet%20EST%20paper%20In%20Vitro%20Tests%208%20Oil%20Dispersants%207-6-2010.pdf*

Alphabetical Index of Projects

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-RTD	European Commission Directorate General for Research and Technology Development
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 screening

JRC	European Commission Joint Research Centre
NRC	United States National Research Council
OECD	Organisation for Economic Co-operation and Development
PBPK	Physiologically-based pharmacokinetic 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

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	Poland	PL
Denmark	DK	Portugal	PT
Estonia	EE	Romania	RO
Finland	FI	Slovakia	SK
France	FR	Slovenia	SI
Germany	DE	Spain	ES
Greece	GR	Sweden	SE
Hungary	HU	Switzerland	CH
Ireland	IE	United Kingdom	UK
Italy	IT	United States	US
Japan	JP		

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 recommend strategic priorities for future EU funding calls.

The main conclusions and recommendations resulting from the first **AXLR8** workshop and from discussions of the **AXLR8** Scientific Panel are as follows:

- Substantial progress has been made in Europe on the development of alternative test methods and integrated testing strategies. Examples include the EU-funded projects Sens-it-iv and ReProTect, which are positioned to deliver new, non-animal tools for the assessment of skin sensitisation and reproductive toxicity, respectively.
- Opportunities exist for exciting cross-fertilisation and creation of synergies among EU research teams and international initiatives such as ToxCast and Tox21 in the United States.
- A coordinated, long-term strategy toward a common goal is urgently needed. Only then can a practical roadmap be developed that integrates new and existing European and global research programmes and defines knowledge gaps and future research needs and priorities, allowing for the creation of a structured framework for transatlantic/pacific coordination and collaboration going forward.
- Recommendations for short-term research priorities are provided.

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** provides the tools for effective real-time dialogue, information exchange, problem solving, and international cooperation.

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