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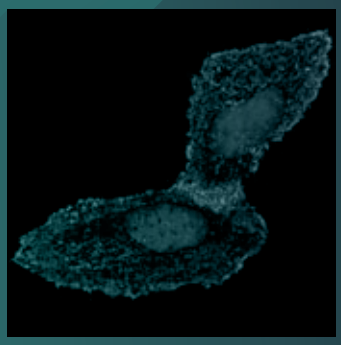
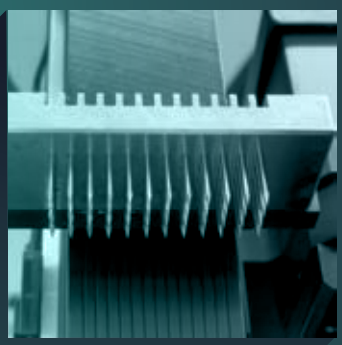
ALTERNATIVE TESTING STRATEGIES

PROGRESS REPORT 2009

Replacing, reducing and refining use of animals in research





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ALTERNATIVE TESTING STRATEGIES

PROGRESS REPORT 2009

Replacing, reducing and refining use of animals in research

Genomics & Biotechnology for Health

Edited by
Eugénia M. Nogueiro

Directorate-General for Research
Life Sciences, Genomics and Biotechnology for Health

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FOREWORD

Dissemination of research results in 'real time' is an important tool in the management of research programmes.

The present book summarises the results of ongoing research work on the 3Rs Principle (Refinement, Reduction, and Replacement of animal tests) funded within the Health theme of the Sixth and Seventh European Research and Technological Development (RTD) Framework Programmes (i.e. FP6 and FP7, respectively) in the context of policy needs, industry requirements and scientific challenges. It focuses on the increase of knowledge achieved over the last two years and gives an outlook to potential future research strategies and priorities. In this sense, the book should be considered a follow-up of the brochure on 'Alternative Testing Strategies – Replacing, reducing and refining use of animals in research'[1] published in 2008.

In order to facilitate and optimise the use of the book, an attempt has been made to group the research projects in line with their respective technologies and approaches. Although the projects originated from various calls for proposals of FP6 and FP7 addressing a number of different applicability areas, they have been grouped under the following headings: cell-based technologies, integrated testing strategies,

1 See ftp://ftp.cordis.europa.eu/pub/fp7/docs/alternative-test-strat_en.pdf online.

-omics, bioinformatics and computational biology, computational modelling and estimation techniques, high throughput techniques, and forums and workshops.

Within individual projects dealing with the development of new assays, an effort has been made to characterise the various test methods in a structured way (by clinical domains, end-points, applicability, their status between basic research and validated test methods, etc.).

The transition from ongoing research work to new promising areas requires in-depth knowledge of the state of the art and excellent communication between the scientists involved. Specific support and coordinating actions such as the FP7 projects START-UP ('Scientific and technological issues in 3Rs alternatives research in the process of drug development and Union politics') and AXLR8 ('Accelerate the transition to a new toxicity pathway-based paradigm for chemical safety assessment') will help focus and streamline future activities. The resulting essential building blocks will create a solid basis for this long-term endeavour.

With the present book, I intend to launch a series of annual 'snapshots' of progress made in ongoing projects funded by the Health programme. It is planned that the annual status reports give an updated outlook to future research strategies, taking into account progress in scientific and technological developments and changes in political requirements.

'Reduction' and 'Refinement' of the use of animals in tests were and are being addressed by numerous short-term RTD projects. They are of particular importance for the implementation of relevant EU policies. Characteristic examples are the implementation of the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) Regulation or the Cosmetics Directive (76/768/EEC). However there is a lack of methods replacing animal tests in the assessment of toxic effects of chronic exposure, and a relatively high consumption of animals in the currently used test methods. Therefore, developing alternatives to existing repeated dose systemic toxicity testing was placed high on the research agenda. In order to tackle this problem, an experts group was created in winter 2008/2009 by the Directorate-General for Research (DG RTD) and The European Cosmetics Association (COLIPA), with the task of drafting a specific research strategy addressing this topic. In order to trigger further discussions among the relevant stakeholders, from industry, academia and regulatory bodies, and to create the bridges between ongoing and future RTD work, the recommendations of the experts group are presented in this book.

I would like to use this opportunity to thank all coordinators, their project partners, and the experts involved for their valuable contributions to this specific topic of the European RTD Framework Programme. In particular, however, I would like to thank Ms Eugénia Marques Nogueiro for her excellent and focused work in the preparation of this book. Without her enthusiasm, it would not have been possible to produce it within such a short time – an essential prerequisite if we have 'annual snapshots' on results in mind.

Ruxandra Draghia-Akli
Director
Health Research

1. INTRODUCTION

Relevant legislation, regulations and directives require, in the medium-to long-term, a phasing out of animal tests, thus putting a great deal of pressure on research, validation and regulatory acceptance of non-animal alternatives.

To boost the implementation of the 3Rs Principle, the subject of alternative testing methods has been part of the European RTD Framework Programmes for more than 20 years. The main contributions to this issue were made by the various Environment and Health Specific Programmes. Health and environment fields share the need for non-animal models for drug development, chemical toxicity and ecotoxicology, and product safety assessment, be it food additives, cosmetics, or any other substance or mixture with potential risks for consumers' health.

Over the last 20 years, the financial EU RTD contribution to these areas amounted to some EUR 200 million.

The Commission has dealt with 3Rs research through a number of initiatives involving different EU services and agencies, including ECVAM, ECHA, the Directorate-General for Enterprise and Industry (DG ENTR), the

Directorate-General for Health and Consumers (DG SANCO), the Directorate-General for the Environment (DG ENV) and DG RTD.

For example, in the context of the implementation of the REACH regulation, which calls for massive testing of industrial chemicals, two main areas of research have been developed, with emphasis on Refinement and Reduction: the one of *in silico* methods, such as mathematical models of quantitative structure-activity relationships (QSARs), and that of intelligent testing strategies.

But the full replacement of animals in safety testing is a challenging long-term target in the implementation of the 3Rs Principle.

1.1 Human safety, products safety

Chemicals have an inherent property of having potential adverse effects on human health (hazard) which can become effective under certain conditions of exposure (amount of intake or one that reaches a target system). For each given substance and product in the market, such as drugs, cosmetics, pesticides, food additives, or their chemical ingredients, a risk assessment has to be carried out in order to determine the risk it poses to human health, namely to specific target systems, i.e. the probability of adverse effects to occur under certain circumstances. This process is needed for regulators to decide about a particular substance security for human health and/or the environment.

The process of risk assessment consists mainly of the following:

- hazard identification: comprehends the determination of the substance or product's intrinsic toxicity, characterised by the types of injury (systemic or contact) and the conditions of exposure (exposure duration and dose range: acute, subacute, subchronic and chronic);

- dose-response assessment: means to analyse the relationship between the levels of exposure and the caused effects (in a sample population) and to infer the impact in the entire population;

- exposure assessment: consists of the determination of the extent of human exposure to a substance or product (including its derivatives);

- risk characterisation: the output of this last step is an estimate of the probability of occurrence of an adverse effect of a substance or product when taken or absorbed by an organism, taking in consideration the information from the previous steps.

Data on chemical hazards to human health, necessary for the hazard identification and dose response assessment, may come from epidemiological studies and poisoning incidents, but in the absence of human data toxicity tests using animals (rodents, primates, cats and dogs amongst other animals) are the most common source of information. This involves the administration of the test substance or product in a single (usually extremely high) dose (acute systemic toxicity), lethal in a short time or causing evident toxicity, or in daily doses (repeated dose toxicity) in order to determine the 'no observed adverse effect levels' (NOAELs) and the adverse effects in the organs or systems after exposures of one-month ('subacute'), three-month ('subchronic'), and/or two-year ('chronic') durations.

Effects observed range from clinical signs of toxicity (e.g. loss of balance and loss of body weight) to skin and eye damage, reversible (irritation) or irreversible (corrosion), developmental and reproductive damage (generally determined

after one or more generations of offspring), genetic mutations and other alterations of the genetic material (genotoxicity), cancer formation (carcinogenicity – through genotoxic or non-genotoxic mechanisms), brain and nervous system damage (neurotoxicity), and adverse effects on the immune system (immunotoxicity).

This empirical approach to identify chemical hazards and assess the dose-response relation, relying on animal models of questionable adequacy to predict human health effects, highly costly and time consuming, and which raises great concerns about animal welfare, is now under the early beginning of a process of change.

New technologies and tools made available after the sequencing of the human genome are expected to allow for the development of a new methodology of retrieving toxicological information based on the understanding of the effects of chemicals at a molecular and cellular level regarding the toxicity endpoints.

1.2 The 3Rs Principle and the legislative framework

An alternative test method to the present methods used in toxicological studies is any method that replaces the use of animals (replacement alternative), uses fewer animals (reduction alternative) or causes least harm to animals (refinement alternative). This so-called 3Rs Principle, formulated by William Russell and Rex Burch, in 1959, in the publication *The Principles of Humane Experimental Technique*, is present in the relevant EU legislation.

In 1986, the Council adopted Directive 86/609/EEC (Animals Directive) on the protection of animals used for experimental and other scientific purposes. Briefly summarised, this directive, which is currently under revision, aims to maximise animal welfare and to reduce to a minimum the number of animals used in experiments for scientific purposes, be it for the development, production and safety testing of drugs, food products or any other chemicals and products, for physiological studies, or for the protection of human health and the environment. Moreover, it seeks to guarantee the welfare of animals used in experiments, as far as general care and accommodation is concerned, and whenever such use is unavoidable.

Also, the REACH Regulation on chemicals and their safe use (EC 1907/2006), concerning the Registration, Evaluation, Authorisation and Restriction of Chemical substances, and covering all substances manufactured and imported, sold on their own or as a component of a product, promotes non-animal tests. The test methods to be applied for this purpose were set by Council Regulation No 440/2008, which updates and replaces Annex V of Council Directive 67/548/EEC, on the classification, packaging and labelling of dangerous substances (Directive on Dangerous Substances). Recital 5 of the Regulation EC 440/2008 states: 'The principles of replacement, reduction and refinement of the use of animals in procedures should be fully taken into account in

the design of the test methods, in particular when appropriate validated methods become available to replace, reduce or refine animal testing.'

In the particular case of cosmetics, Council Directive 76/768/EEC (Cosmetics Directive), through its seventh Amendment (Directive 2003/15/EC of the European Parliament and of the Council), introduced testing and marketing bans to the cosmetic industry with the aim of phasing out animal testing. It established a prohibition to test finished cosmetic products and cosmetic ingredients on animals (testing ban), and a prohibition to market in the European Community, finished cosmetic products and ingredients included in cosmetic products which were tested on animals (marketing ban).

The testing ban on finished cosmetic products has been applied since 11 September 2004. However for six years after entry into force of the directive, i.e. until 11 March 2009, the testing ban on their ingredients and combinations of ingredients, as well as the marketing ban, were applied step by step, at the same time as alternative methods were validated and adopted in EU legislation with due regard to the OECD validation process. For the marketing ban of substances or products tested in animals for human health effects of repeated-dose toxicity, reproductive toxicity and toxicokinetics, a deadline of 10 years after entry into force of the directive was foreseen, i.e. 11 March 2013, irrespective of the availability of alternative non-animal tests.

As recognised by Recital 5 of Directive 2003/15/EC, 'the safety of cosmetic products and their ingredients may be ensured through the use of alternative methods which are not necessarily applicable to all uses of chemical ingredients' and so, to supplement Annex V of the Directive on Dangerous Substances, Annex IX was created to ensure the regulatory acceptance of alternative methods that would only be applicable to the cosmetic sector but not to the whole chemical sector.

1.3 Often-expressed concerns about the replacement of animals in scientific experiments

The revision in progress of the Animals Directive aims to strengthen the protection of animals used in scientific experiments in line with the European Union's Protocol on Animal Welfare, to level Member States' standards for experimental animals and to enhance the quality of research conducted in the European Union (see http://europa.eu/rapid/pressReleasesAction.do?reference=IP/08/1632&format=HTML&aged=0&language=EN&guiLanguage=en).

A great number of concerns have been raised by the Members of the European Parliament, scientific institutions and general public regarding the revision of this directive for the protection of animals used in scientific experiments. Questions and answers on this subject can be found online (http://europa.eu/rapid/pressReleasesAction.do?reference=MEMO/08/677&format=HTML&aged=0&language=EN&guiLanguage=en).

The main concern linked to the full implementation of the 3Rs Principle are located in the triangle of animal welfare and ethics, industrial competitiveness and scientific/technological progress in Europe.

The use of human embryonic stem cells

The FP7 decision [2] explicitly excludes from Community funding, research activities intended to create a human embryo solely for the purpose

2 Decision No 1982/2006/EC of the European Parliament and of the Council of 18 December 2006, concerning the Seventh Framework Programme of the European Community for research, technological development and demonstration activities (2007-2013).

of research or for the purpose of stem cell procurement, including by means of somatic cell nuclear transfer.

Furthermore, the decision whether or not to allow research on human stem cells remains a matter for the Member States. No activity will be funded in a Member State where such activity is forbidden.

Before approval, research proposals involving the use of hESC must go through stringent ethical reviews. The proposal is assessed by at least two independent ethical reviews: one in the country itself where the research will be carried out and one organised by the European Commission. It is then presented for approval, on a case-by-case basis, to the Member States, meeting as a Regulatory Committee. No project involving the use of hESC will be funded that does not obtain approval from the Member States. In particular cases, an ethical review may be carried out during the lifetime of the project.

Scientific progress and industry competitiveness

New or alternative testing methods should be at least as predictive as the methods currently used. Faster, cheaper and more reliable methods would constitute real progress in safety assessment and a significant increase in the competitiveness of European industry.

Furthermore, it can be expected that the process of developing such alternative methods will demand innovative techniques, generate technological progress, and result in an overall increase of knowledge, opening new doors for science and business. Therefore, today's investment in research can represent a competitive advantage for Europe in the future.

The European Union is not alone in the conviction that the implementation of the 3Rs Principle is a fundamental process. In 2007, the United States National Research Council (NRC) of the National Academies, commissioned by the United States Environmental Protection Agency's (US EPA), published the report 'Toxicity Testing in the 21st Century: a Vision and a Strategy'. This envisions a new paradigm to address risk assessment, based on the identification and evaluation of toxicity pathways, and has lead to EPA's strategic plan for evaluating the toxicity of chemicals [3], announced at the beginning of 2009. It advocates a new concept, which is expected to 'create more efficient and cost-effective means to screen and prioritize for further assessment the tens of thousands of chemicals that are already found in the environment. The new paradigm should facilitate evaluating the susceptibility of different life-stages and genetic variations in the population, understanding the mechanisms by which toxicity occurs, and considering the risks of concurrent, cumulative exposure to multiple and diverse chemicals, while at the same time significantly reducing reliance on animal testing for assessing human risk.'[4].

3 The report 'The U.S. Environmental Agency's Strategic Plan for Evaluating the Toxicity of Chemicals' (March 2009) is available online (http://www.epa.gov/OSA/spc/toxicitytesting/docs/toxtest_strategy_032309.pdf).

4 See http://www.epa.gov/OSA/spc/toxicitytesting/ online.

1.4 Institutions and organisations in the field of safety assessment and alternative testing methods

At European level

▸ IHCP

The Institute for Health and Consumer Protection (IHCP) is one of the seven scientific institutes of the Joint Research Centre (JRC) Directorate-General of the European Commission, and is located in Ispra, Italy. The IHCP works for the protection of the interests and health of the consumer in the framework of EU legislation on chemicals, food and consumer products, providing scientific and technical support to Commission Services and to Member States authorities concerning chemicals testing methods.

The IHCP consists of the following five scientific units:

1. Chemical assessment and testing
2. *In vitro* methods
3. Molecular biology and genomics
4. Nanosciences
5. Systems toxicology.

The status of alternative methods in Europe, from scientific protocols submitted for pre-validation to active use in a regulatory context, can be tracked on the TSAR website (http://tsar.jrc.ec.europa.eu/) of the European Commission.

More information about IHCP is available online (http://ihcp.jrc.ec.europa.eu/).

▸ ECVAM

The JRC Institute for Health and Consumer Protection (IHCP) hosts the European Centre for the Validation of Alternative Methods (ECVAM).

ECVAM supports the European Commission's aim to replace animal tests wherever possible, as well as to reduce the actual number of animals used by refinement of tests that cannot immediately be replaced. Its main task is to coordinate at EU level the scientific validation of alternative methods that afterwards feed into the regulatory acceptance decision process. In this role, ECVAM acts as a focal point for the exchange of information on the development and validation of alternative methods and manages a database service on alternative approaches.

ECVAM is also involved directly at the research level in the elaboration of integrated testing approaches (based on other means than animal tests) to provide more accurate and robust scientific data for risk decision analysis. A particular focus is on rapid toxicity screening of chemicals in support of the Cosmetics Directive and the REACH legislation.

Due to the political sensitivity of its work, ECVAM has its own Scientific Advisory Committee (ESAC), comprising representatives of the Member States, relevant industrial associations, academic toxicologists, animal welfare organisations and other Commission services.

More information about ECVAM can be found online (http://ihcp.jrc.ec.europa.eu/).

ECHA

The European Chemicals Agency (ECHA) manages the technical, scientific and administrative aspects of REACH, ensuring consistency at Community level regarding the safe use of chemicals, and the competitiveness of European industry.

More information about ECHA can be found online (http://echa.europa.eu/home_en.asp).

EPAA

The European Partnership for Alternative Approaches to Animal Testing (EPAA) is a joint initiative between the European Commission, 7 industry associations and more than 30 individual companies. It was launched in November 2005 by the European Commission Vice President Günter Verheugen and the Science and Research Commissioner Janez Potočnik.

EPAA's purpose is to promote the development and implementation of the alternative testing methods in safety testing. This will be done through the dissemination of information about existing methods for replacement, reduction and refinement of the use of animal tests – and about research programmes, the support for the development of new alternative approaches and testing strategies, and the promotion of the acceptance of alternative testing methods by regulators.

More information about EPAA can be found online (http://ec.europa.eu/enterprise/epaa/index_en.htm).

ecopa

The European Consensus-Platform for Alternatives (ecopa) aims to stimulate research on alternatives to animal experiments and to promote the acceptance of alternatives in experimental practice. ecopa promotes consensus discussions with all relevant stakeholders, i.e. animal welfare, industry, academia and governmental institutions in various countries, through its membership platforms.

More information about ecopa can be found online (http://www.ecopa.eu/).

International initiatives

ICCVAM and NICEATM

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in 2000 in the United States with the purpose of promoting the development, validation, and regulatory acceptance of new, revised, and alternative regulatory safety testing methods. ICCVAM comprises representatives from 15 federal agencies that generate or use toxicological data in the context of health and safety of people, animals, and the environment.

The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) is part of the United States National Institute of Environmental Health Sciences (NIEHS) and holds the responsibility of providing scientific support for ICCVAM. This is done through the coordination of the ICCVAM meetings and its subcommittees and interagency working groups, the evaluation of test methods submissions and nominations, the preparation of technical review documents, and the organisation of scientific workshops and independent scientific peer review meetings.

More information about ICCVAM can be found online (http://iccvam.niehs.nih.gov/about/about_ICCVAM.htm).

More information about NICEATM can be found online (http://iccvam.niehs.nih.gov/about/about_NICEATM.htm).

▸ OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation with representatives of 30 industrialised countries in North America, Europe and the Pacific, as well as the European Commission.

In its Environment, Health and Safety Programme, the OECD provides information on internationally approved testing methods used by government, industry and independent laboratories to assess the safety of chemical products ('Guidelines for the Testing of Chemicals'[5]).

The OECD also issues guidance documents on the validation and international acceptance of new or updated test methods for hazard assessment[6]. Non-animal alternative testing methods which meet the OECD's regulatory safety requirements include tests on skin corrosion, phototoxicity and skin absorption.

Addressing the specific topic of Quantitative Structure-Activity Relationships ((Q)SARs), the OECD (Q)SAR Project has published the Guidance Document on the validation of (Q) SAR Models [7] and is developing a 'Toolbox' for practical applications of (Q)SARs in specific regulatory contexts by governments and industry.

More information about OECD Environment, Health and Safety Programme is available online (http://www.oecd.org/dataoecd/18/0/1900785.pdf).

5 See http://masetto.sourceoecd.org/vl=391653/cl=12/nw=1/rpsv/periodical/p15_about.htm?jnlissn=1607310x online.

6 See http://www.olis.oecd.org/olis/2005doc.nsf/LinkTo/NT00002EAE/$FILE/JT00188291.PDF online.

7 See http://www.oecd.org/dataoecd/33/37/37849783.pdf online.

2. PROJECTS SUPPORTED BY THE EU HEALTH PROGRAMME

The projects supported by the EU Health programme presented 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' projects are exclusively supported by the funding scheme of Specific Support Actions (SSA). These cover training, conferences or prospective studies in support of the programme.

However, the remaining projects are more difficult to group, as in some cases they use approaches falling under more than one of the topics above. This is particularly the case of Integrated Projects

Table 1. Overview of FP6 and FP7 projects on 'Alernative Testing Strategies'.

Projects' technologies and approaches	Projects	Framework programme	Funding scheme
1. 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
2. Integrated testing strategies	ACuteTox	FP6	IP
3. -omics, bioinformatics and computational biology	Predictomics	FP6	STREP
	Sens-it-iv	FP6	IP
	carcinoGENOMICS	FP6	IP
	PREDICT-IV	FP7	IP
4. Computational modelling and estimation techniques	OpenTox	FP7	STREP
5. High throughput techniques	TOXDROP	FP6	STREP
	COMICS	FP6	SME-STREP
	NanoTEST	FP7	STREP
6. Forums and workshops	CONAM	FP6	SSA
	SCARLET	FP6	SSA
	ForInViTox	FP6	SSA
	InViToPharma	FP6	SSA
	START-UP	FP7	SSA

(IPs), the largest-sized projects, which adopt a multidisciplinary approach, for example by combining the development or improvement of *in vitro* cell models with genomics, proteomics, metabolomics, (Q)SAR analysis, and/or biochemical pathway analysis. Nevertheless, some overlapping of subjects also occurs among the Specific Targeted Research Projects (STREPs) and SME-Specific Targeted Research Projects (SME-STREPs), which are smaller projects focusing on specific research issues, and so are less multidisciplinary and wide ranging than IPs.

As regards the participation of small to medium-sized enterprises (SMEs), these have been specifically encouraged to participate in selected FP6 calls, together with universities, research centres and other industries and industrial associations, because of their recognised key role in the health and biotech sectors. As a result, in the field of research on alternative testing methods, almost 25% of the projects are supported by the SME-STREP funding scheme, where research-led SMEs play a leading role.

Table 1 gives an overview of the projects according to their common features. An additional SSA project not included in this section but mentioned in 'Defining the state of the art' (START-UP) is also included in the table.

2.1 Cell-based technologies

Eight projects focus their work on developing *in vitro* models capable of replacing animals in toxicity tests: ReProTect, VITROCELLOMICS, MEMTRANS, EXERA, INVITROHEART, LIINTOP, ARTEMIS and ESNATS.

EXERA targets the development of two-dimensional (2D) and three-dimensional (3D) cell assemblies to be used as *in vitro* models.

VITROCELLOMICS, INVITROHEART, and ARTEMIS also have the objective of developing 3D *in vitro* models to mimic selected elements of organs for *in vitro* studies on susceptibility to xenobiotics.

ReProTect, MEMTRANS, LIINTOP and ESNATS focus on the development of assays and endpoints for improved *in vitro* models to predict toxicity.

ReProTect

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

Objectives

ReProTect assembles 33 different European partners from Academia, Industry including small to medium-sized enterprises (SMEs) and Governmental Institutes. Due to the Registration, Evaluation and Authorisation of Chemicals (REACH) Regulation and the seventh Amendment of the Cosmetic Directive implementation, 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 ~60% of the total costs of the REACH exercise will be with reproductive toxicity studies. On the other hand, the new European legislation itself asks for the use of alternative methods rather than for the application of conventional testing designs for the thousands of chemicals to be tested. For this reason, the integration of alternative tests in a testing strategy 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 approaches) is strongly encouraged. On this background, the overall aim of ReProTect is to develop new *in vitro* models and to improve existing ones, in order to include them into a testing strategy, which aims to provide detailed information on the hazard of compounds to the mammalian reproductive cycle.

Experimental design

In order to cover the fundamental aspects of mammalian reproduction, the reproductive cycle was broken down in its biological components, covering key aspects such as (1) male and female fertility, (2) implantation, and (3) prenatal development. Furthermore, a fourth research area 'Cross-Cutting Technologies' has been

created in order to explore the applicability of innovative methods. The general structure of the ReProTect project is illustrated in Figure 1.

A series of workshops were organised in order to identify the most promising models to be included in the project, and detailed reports on their outcome are published (for more information we refer to the references section).

In the area of female/male fertility and implantation, adverse effects on mammalian reproduction are identified by various promising tests reflecting different toxicological endpoints such as: Leydig and Sertoli cells (Leydig and Sertoli cell toxicity assays); folliculogenesis (FBA assay); germ cell maturation (FBA assay and bovine *in vitro* maturation); motility and morphology of the sperm cells (CASA assay); sperm DNA damage (ReProComet assay); steroidogenesis (FBA and Granulosa assay); the endocrine system (MELN, CALUX, PALM and PANVERA); fertilisation (mouse and bovine *in vitro* fertilisation assays); the pre-implantation embryo (mouse and bovine *in vitro* pre-implantation assay); placentation (chorionic villi and pericyte cell culture, placenta perfusion, trophoblast cell assay); and uterus function (endometrial/endothelial/stromal cells and explant-based assays).

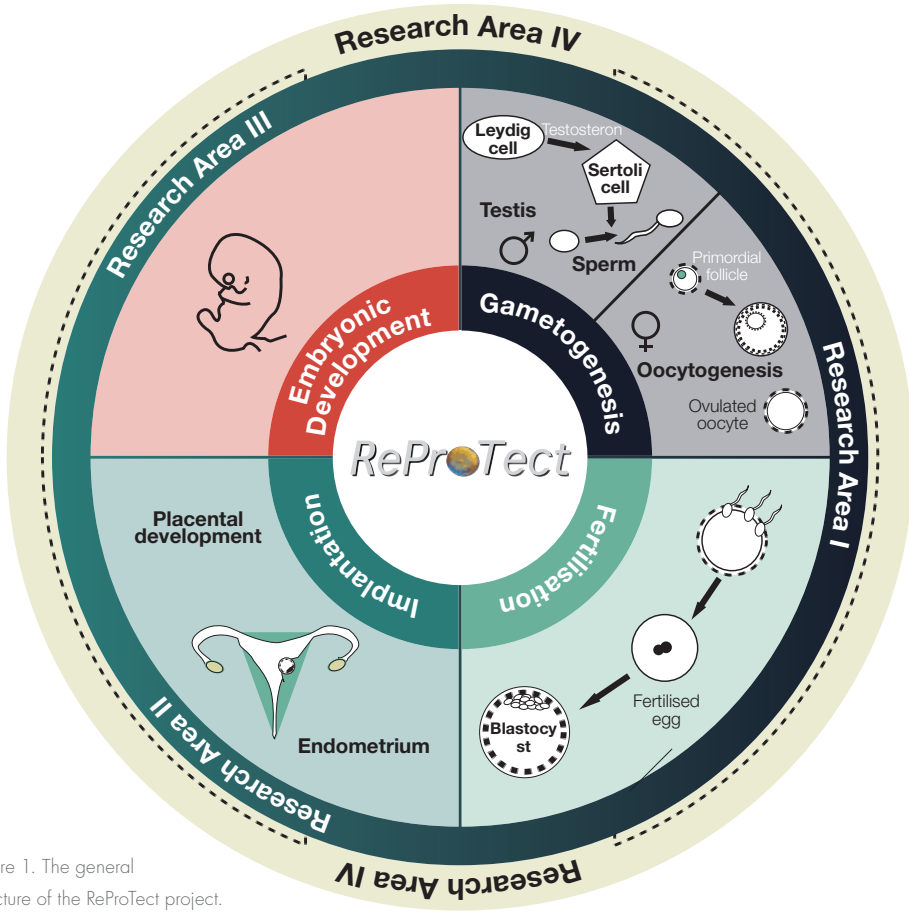

Figure 1. The general structure of the ReProTect project.

For detection of adverse effects on prenatal development, in addition to the whole embryo culture, the potential of an already existing test using murine (mES) stem cells (embryonic stem cell test, EST) has been further investigated by two laboratories. In addition, ReProTect was the first integrated project that included human embryonic stem (hES) cells in an alternative assay to identify toxicants targeting neuronal development. The use of hES cells is a unique approach aiming to avoid misclassification of chemicals because of inter-species variations in toxic response. The utility of proteomics to develop a specific protein signature predictive of embryotoxicity of chemicals has also been exploited. Proof of principle for a metabolic activation system able to identify pro-teratogenic compounds in the mES embryotoxicity test has been established.

A formal validation of the different project tests was not the aim of ReProTect. Nevertheless, in order to speed up future validation, the development/optimisation of each assay followed the ECVAM Modular approach and for some tests the transferability of the method has already been assessed in a second laboratory. After a preparation of a complete dossier, the methods will be externally evaluated for their performance in order to identify the most promising ones ready for entering in a formal validation study (Figure 2).

A 'technology scout' was established in order to retrieve already existing information on alternative technologies in the field of embryotoxicity testing.

Finally, progress has been achieved concerning the implementation of cross-cutting, mechanism-based approaches as tools for testing and risk assessment. Quantitative structure activity (QSAR) models have been developed to predict placenta transfer of chemicals, their potential endocrine disruptor activity and their patterns of metabolite formation. The use of toxicogenomics integrated with functional parameters ('phenotypic anchoring') to assess relevance and consistency of gene expression changes has been explored

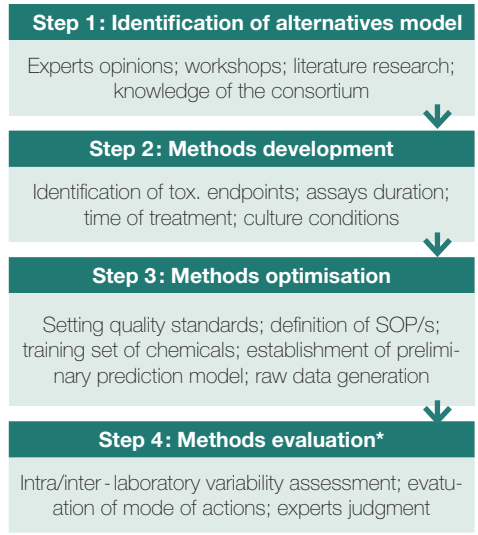

Figure 2. Development/optimisation of tools for reproductive toxicity assessment in ReProTect.

as regards receptor-mediated and differentiation pathways. Special progress has been obtained in the field of endocrine disruption where different assays on transactivation of oestrogen receptor alpha and androgen receptor have been developed and optimised, and data on transferability have been obtained.

For an introduction into the ReProTect project a brochure was developed, which informs the interested reader about the projects' scientific research field. In addition, a project webpage was set up in order to make the projects' achievements publicly available (www.reprotect.eu).

Results

Within the last five years, ReProTect explored the predictive power of a range of pioneering *in vitro* tests. At the basis stood the search for suitable test chemicals, and an extensive screening including ~500 compounds was performed in order to identify the most relevant ones to be used in test definition. More than 130 peer-reviewed reproductive toxicants with different toxicological mechanisms were ultimately selected and tested in order to support the optimization process of test protocols developed in ReProTect. The pool of substances defined was externally evaluated in order to collect all relevant information on their adverse effects on female/male fertility, implantation and embryonic development.

A consolidated Standard Operating Procedure (SOP) is now available for each individual assay which was challenged during the testing phase. Furthermore, in order to ensure a transparent evaluation on the performance of the developed tests, an external independent statistical evaluation is currently under way. The statistical analysis assesses the intra-laboratory variability of the assays and for some tests (e.g. for some of the endocrine disruptor assays) the inter-laboratory variability. The results of the statistical evaluation, together with the supporting scientific information on the tests, will form the base for a later experts' judgment that will allow the identification of the most promising tests which could then enter into formal validation.

In research area I (fertility), a total of 15 *in vitro* tests were developed/optimised which are able to detect adverse effects on (1) mature sperms, (2) folliculogenesis, (3) steroidogenesis, (4) oocytes maturation and fertilisation, and finally (5) the pre-implantation embryo. Some promising results have been obtained with the *in vitro* oocytes maturation/fertilisation (IVM/IVF) assays. The two tests demonstrated that they are able to identify adverse effects of chemicals on the process of oocytes maturation and fertilization (Figure 3). The developed SOP was challenged by testing 15 well-known chemicals and the intra-laboratory variability showed a good reproducibility of the methodologies. The data produced have been published. Due to the successful development of the IVM test it was decided to investigate its transferability to a different laboratory. For this reason, a new project partner was recruited in 2008. The analysis of data generated by the new partner will allow the assessment of the inter-laboratory variability of the method.

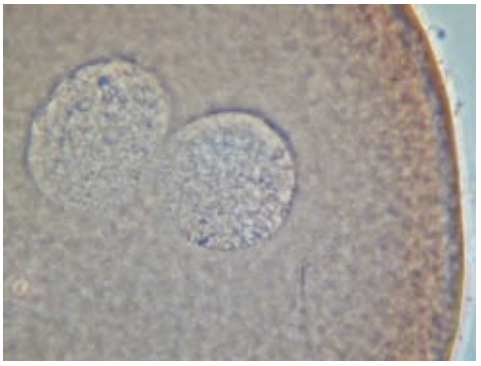

Figure 3. Female and male pronuclei with sperm tail in a bovine zygote stained with Lacmoid solution.

Another test that showed good performance was the Follicle Bioassay (FBA). FBA is a multiple endpoints methodology which mimics *in vitro* aspects such as folliculogenesis, steroidogenesis and maturation of the murine oocytes (Figure 4). Twenty different chemicals/drugs have been tested demonstrating the capacity of such complex assay to identify adverse effects on female fertility. The assessment of its intra-laboratory variability is on the way and the results will be published soon.

The work in research area II (implantation) started as planned in year three of ReProTect after the new partners of this research area had been selected and taken up into the Consortium. In this research area, the focus is on human tissues and tissue explants. The first important biological tissue is the endometrium where chemicals might perturb the process of implantation and/or

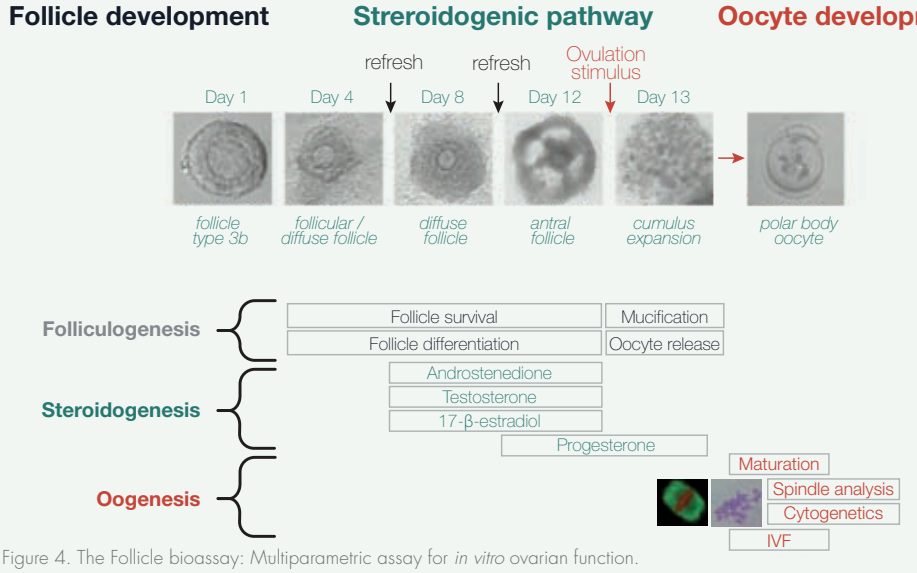

Figure 4. The Follicle bioassay: Multiparametric assay for *in vitro* ovarian function.

cause other reproductive disorders. The results obtained with endometrial explants have shown that the progesterone antagonist RU486 elevated the mRNAs for certain hormones (LIF), while no clear effects were found on the mRNAs for the progesterone or the oestrogen receptors. Yet another potential target tissue is represented by vascular capillary endothelial cells, which have been proven sensitive to environmental toxicants. Being a primary tissue, drug metabolising enzymes can potentially still be expressed in endometrial explants. Main results are that different cytochrome P-450s are specifically expressed, especially in the vascular endothelium and in endometrial gland epithelium.

The second important tissue that was studied in research area II was human placenta. Two groups have independently studied the transfer of a small collection of different compounds in an *ex vivo* human placental perfusion system, with special emphasis on carcinogens such as benzo(a) pyrene and PhIP, phytooestrogens, hormones and endocrine disrupters. The inter-laboratory reproducibility of this system will be evaluated and it is expected that the perfusion system can be regarded as pre-validated by the end of the ReProTect project. Another approach is to study toxic effects especially of endocrine disrupters on hormone release from placental villi explants grown in culture, and on cells (pericytes from vessels) isolated from placentas being exposed to a drug used against epilepsia (valproic acid).

In general terms, SOPs have been developed for all the different human *ex vivo* systems investigated in research area II. The results obtained were somewhat disenchanting, however, since it turned out that the variability in results obtained with individual tissue samples from different donors was often very large and therefore obscuring potential effects of test chemicals. The circular changes in cellular and tissue-function during the menstrual cycle contributes to the difficulty of studying endometrial tissues. For this reason, three of the groups have initiated work with established cell lines such as BeWo choriocarcinoma cells and human endometrial adenocarcinoma Ishikawa cells.

In research area III (Prenatal Development), the development of assays to predict embryotoxicity of chemicals using mouse and human ES cells has been completed. While development into beating cardiomyocytes is the endpoint in the

validated EST – SOPs for additional endpoints, namely neuronal and skeletal differentiation are now available, as well as a preliminary SOP for neuronal differentiation of hES cells. Thirteen additional chemicals were tested by two different laboratories in the validated EST in order to enlarge the assay's database and to better define its applicability domain. The results obtained demonstrated that the prediction model of the validated test has to be modified. Starting from the new data produced in ReProTect, the limitations of the EST as well as potential possible improvements of the assay were discussed during a recent ReProTect/ECVAM workshop, and a report on its outcome will be available to the public in the very near future. State of the art proteome analyses were conducted with extracts from mES and hES cells that were treated with selected chemicals during their neuronal and cardiac differentiation. The promising results obtained will be published soon and the patenting of the methodology is ongoing.

A major problem of all present *in vitro* assays, the lack in capacity to metabolize and thereby bioactivate foreign compounds, was also addressed in two assays, the EST and the whole organ culture system. A metabolic system has been successfully added to the ES cell test, which consists in a pre-incubation step of the test compounds with primary rat hepatocytes, and a proof of principle for this procedure has been achieved for two selected chemicals. This system may be applicable to other *in vitro* systems as well.

A second inherent very obvious problem of all *in vitro* tests is that effects related to pharmacokinetic properties of test chemicals cannot be investigated. This is one of the reasons why *in vitro* tests can only be used for hazard identification but not to establish dose-response relationships. One of the partners has addressed this issue and has used pharmacokinetic models (PBPK) to extrapolate *in vitro* effects seen with the EST to *in vivo* exposure levels. The most encouraging results have been published. A similar study based on results obtained with the bovine spermatozoa cytotoxicity (CASA) test is presently underway.

A major focus in research area IV (Cross-cutting technologies) was on assay systems that are able to detect endocrine disrupting properties of chemicals. QSARs for receptor binding have been optimised; SOPs for toxicogenomic signature analysis matched with phenotypic anchoring in human prostate cell lines are available, targeting chemicals interacting with androgen receptor pathways. In particular, ReProTect has made several important achievements in the field of receptor transactivation assays: the so-called PANVERA test has been optimized within ReProTect and has gone into its formal validation conducted under the umbrella of the OECD in an international collaboration with the USA and Japan; the SOP for a second test, the so-called MELN assay has been transferred to the OECD in order to evaluate its possible inclusion in a test guideline.

A huge database has been established which now combines detailed information on the presumed biological mechanism of action of the numerous test chemicals that were used in the ReProTect assays with the results from the statistical evaluation of data obtained, yielding information on EC50-values and their variances for all chemicals that were used. This database not only allows retrieving a graphical overview of all the results obtained for a given chemical but also permits a rapid comparison of results obtained with the various test systems.

In January 2009, the project consortium decided to initiate a 'feasibility study' in order to challenge a battery of *in vitro* assays that were developed or optimised in ReProTect. A workshop was organised in order to identify the most advanced tests ready to enter into this exercise. Some experts were invited and given the task to define a set of 10 coded chemicals that will be tested in a blinded manner in 10 different ReProTect core tests, which cover aspects related to male/female fertility and embryotoxicity. The preliminary

results of the blinded testing should be ready by the middle of 2009 and the outcome is planned to be published in early 2010 in a 'Special Issue' of Reproductive Toxicology. Due to the additional, initially unforeseen, tasks the duration of the project was prolonged for six months and the project is now planned to end by December 2009. A summary of the methods developed within ReProTect is presented in the following tables (Tables 1 to 25).

Table 1

Name of the test method	CASA test
Clinical endpoint	Male fertility toxicity.
Cell (line)	Primary bovine spermatozoa.
Method description	Adverse effects of chemicals on bovine spermatozoa (changes in motility) with possible implications on fertility potential are monitored by a computer assisted sperm analysis system (CASA).
SOP	Yes.
Endpoints	A dose range finding test (DRF) is performed. The IC50 (total motility of sperm is 50% of the control value) is calculated. The main study is performed with concentrations of test chemicals close to the calculated IC50. Velocity, motion and viability of bovine sperm are analysed in real time, allowing the detection of chemical-induced changes in sperm motility.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability has been assessed.

Table 2

Name of the test method	ReProComet Assay
Clinical endpoint	Male fertility toxicity.
Cell (line)	Primary bull spermatozoa.
Method description	The method is based on a modification of the comet assay to detect chemically induced DNA strand breaks and alkali labile sites in bull sperm. Mature sperms are essentially devoid of repair enzymes and, as such, cannot be applied in the classical comet assay. In the ReProComet assay the addition of a crude protein extract from HeLa cells provides repair enzymes that recognise DNA adducts and produce detectable DNA breaks.
SOP	Yes.
Endpoints	(1) Tail length (2) Fraction of tail and head DNA (3) Tail moment (fraction of migrated DNA multiplied by the tail length). Brightness and length of comet tails correlate with the extent of DNA damage.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 3

Name of the test method	Leydig cell tox test (based on genetically engineered cells)
Clinical endpoint	Male fertility toxicity.
Cell (line)	BLT1 mouse Leydig cell line genetically modified.
Method description	Leydig cells are the major male steroid hormone producing cells and of essential importance in male reproduction. Leydig cells in culture (BLT1 mouse Leydig cell line) are treated with β-hCG to stimulate steroid hormone output, while being exposed to various concentrations of a test compound.
SOP	Yes.
Endpoints	Progesterone and testosterone; MTT for general toxicity.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 4

Name of the test method	Sertoli cell tox test
Clinical endpoint	Male fertility toxicity.
Cell (line)	Primary rat Sertoli cells and a rat Sertoli cell line, (SerW3)
Method description	Sertoli cells are somatic cells present in the seminiferous tubules. They influence spermatogenesis. Two culture systems are applied: (1) Primary rat Sertoli cells and (2) a rat Sertoli cell line, (SerW3). The test intends to assess testicular toxicity of test compounds based on Sertoli cell toxicity with the best prediction compared to *in vivo*.
SOP	Yes.
Endpoints	(1) Cytotoxicity (MTT assay) (2) Secretion of Inhibin B (ELISA): Alteration in EC50 values reflect alteration of Sertoli cells functions due to test chemical's toxicity.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability has been assessed.

Table 5

Name of the test method	*In vitro* bovine fertilisation test (bIVF)
Clinical endpoint	Fertility toxicity.
Cell (line)	Mature bovine oocyte and bull sperms.
Method description	The bIVF assay focuses on the use of bovine oocytes and sperms for toxicity testing during the process of fertilisation *in vitro*. Purpose: Test to (1) screen for adverse effects of chemicals on the process of oocyte fertilisation and (2) investigate the mechanism of action of reprotoxic compounds. Both oocytes and sperms are exposed to test chemicals; therefore, the adverse effects on the function of both gametes can be monitored.
SOP	Yes.
Endpoints	(1) Penetration of capacitated bull spermatozoa into matured oocytes (2) Formation of the female and male pronuclei.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 6

Name of the test method	*In vitro* bovine maturation test (IVM)
Clinical endpoint	Female fertility toxicity.
Cell (line)	Immature bovine oocyte.
Method description	The IVM assay focuses on the use of bovine oocytes for toxicity testing during the process of oocyte maturation *in vitro*. The test screens for potential adverse effects on the process of oocyte maturation after exposure of cumulus-oocyte complexes to test substances, with special reference to nuclear configuration changes within the oocyte as compared to control non-exposed oocytes.
SOP	Yes.
Endpoints	Successful achievement of the maturation stage metaphase II (completion of meiosis up to the metaphase II).
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability assessment is in progress.

Table 7

Name of the test method	*In vitro* bovine pre-implantation test
Clinical endpoint	Adverse effects on early embryonic development.
Cell (line)	Mature bovine oocyte and bull spermatozoa.
Method description	*In vitro* fertilized oocytes are exposed to test chemicals for four days before the nuclear number of the embryo is evaluated under the microscope. The relevant toxicological endpoint is the transition between the 8 and the 16 cell stage (initiation of the 4th cell cycle) that represents the maternal-embryonic transition and the activation of the embryonic genome. Purpose: To monitor potential effects of test chemicals on the developmental process of *in vitro* produced zygotes with special reference to transcriptional activation of the embryonic genome.
SOP	Yes.
Endpoints	EC50 values (inhibition of the initiation of the 4th cell cycle in 50% of the cleaved embryos).
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Positive control	Cycloheximide.
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 8

Name of the test method	Mouse embryo bioassay (MEA)
Clinical endpoint	Female fertility toxicity.
Cell (line)	Mouse zygote after *in vivo* fertilisation.
Method description	MEA is a long-term multiparametric bioassay that allows the *in vitro* development of mouse embryos for eight days. Mouse zygotes are cultured under defined standardised conditions accompanied by daily evaluation and scoring of embryos. Purpose: (1) Screening of compounds for adverse effects on pre-implantation embryo development and survival (NOEL determination; ranking of compounds) (2) Determination of the sensitive stage(s) (NOEL for each window of development during embryo development) (3) Mechanistic study to reveal the mode of action of compounds interfering with pre-implantation embryo development.
SOP	Yes.
Endpoints	Fertilisation/ first cleavage (2-cell stage); cleavage (more cell stage); compaction (morula stage); embryo differentiation and development (blastocoel stage, blastocyst stage, hatched blastocyst stage); embryo viability.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 9

Name of the test method	Granulosa cells steroidogenesis test
Clinical endpoint	Female fertility toxicity.
Cell (line)	Murine granulosa cell line NT-1 and a clonal variant named NT-1-hAROM (expressing the human aromatase gene).
Method description	This test system studies how chemicals affect the basal levels of steroidogenesis in an immortalized genetically modified murine granulosa cell line. Purpose: Testing of chemicals for effects on steroidogenesis in granulosa cells, one component of the follicle complex. Effect on progesterone production: NT-1 cells are incubated with test compounds and progesterone is measured in the supernatant by immunoassay; cells are then processed for cytotoxicity testing (MTT assay). Effect on estradiol production: NT-1-hAROM cells are incubated with the aromatase substrate androstenedione. Estradiol is measured in the medium by immunoassay and the cells processed for cytotoxicity testing (MTT assay).
SOP	Yes.
Endpoints	(1) cytotoxicity (MTT), (2) de novo progesterone production, (3) conversion of androstenedione to estradiol.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 10

Name of the test method	Follicle bioassay (FBA)
Clinical endpoint	Female fertility toxicity.
Cell (line)	–
Method description	The FBA is a long-term multiparametric bioassay that mimics the ovarian physiology (folliculogenesis, oogenesis and steroidogenesis) *in vitro*. Mouse ovarian pre-antral follicles are grown *in vitro* up to the preovulatory stage followed by *in vitro* ovulation induction and mature oocyte retrieval. Follicles develop as *in vivo* with theca cell proliferation, granulosa cell proliferation and differentiation, meanwhile supporting oocyte growth and maturation. During *in vitro* folliculogenesis the steroidogenic pathway is temporally activated and steroid production (androgens, estrogens and progestins) follows the same secretion profile as *in vivo*. The *in vitro* growing follicles are exposed to chemicals either acutely or chronically and effects on folliculogenesis, steroidogenesis and oogenesis are analysed by use of morphological, biochemical and functional parameters. Purpose: Test (1) for screening of compounds potentially producing adverse/proactive effects on ovarian function (2) for screening for endocrine disruptors in 'natural endocrine background' (3) to reveal the mode of action of compounds interfering with female health and fertility capacity.
SOP	Yes.
Endpoints	Ovarian function: -Folliculogenesis: follicle survival, differentiation and responsiveness (ovulation induction) -Steroidogenesis: androstenedione/ testosterone; estradiol; progesterone -Oogenesis: oocyte yield; diameter; nuclear maturation; developmental competence. Concentration-response studies reveal NOEL /LOELs.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 11

Name of the test method	Human endometrial explants
Clinical endpoint	Adverse effects on implantation.
Cell (line)	Primary human endometrial cells.
Method description	A hormonally primed, receptive endometrium is essential for successful implantation of the embryo. Explant cultures are a closer approach to *in vivo* conditions compared to primary or permanent cell cultures and thus suited as a culture model to detect endometrium-related adverse effects of chemicals on implantation. Endometrial tissues from the proliferative and secretory phase are obtained by aspiration curettage from premenopausal women without hormonal treatment for three months. Small tissue pieces are treated with test chemicals and toxicological endpoints are assayed by quantitative RT-PCR, EIA, Western blotting and immunohistochemistry.
SOP	Not yet available.
Endpoints	Steroid receptors (PRα, ERα), LIF, integrin αvß3, calcitonin, IGFBP-1, COX-2, PGDH, CRH-receptor 1, VEGF-A, VEGF receptor 2 (KDR).
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 12

Name of the test method	Human placental chorionic villous explants
Clinical endpoint	Adverse effects on implantation.
Cell (line)	–
Method description	Unlike isolated trophoblast cells, chorionic villous explants have the advantage of preserving the topology of intact villi. Thus, they reflect functional activities of the trophoblast such as invasiveness, proliferation and differentiation. Placental chorionic villi from first trimester human placenta are gently dissected under a microscope and placed on culture dish inserts pre-coated with Matrigel. The inserts are cultured in a serum-free medium and treated with chemicals at the conditions (concentrations and times) defined by the previous analysis of trophoblast-derived cell lines. Purpose: To investigate the effect of chemicals on human trophoblast implantation and development.
SOP	Yes.
Endpoints	Trophoblast functionality parameters: (1) proliferation; (2) differentiation into an invasive form and syncytialization; (3) secretion of hormones, cytokines and growth factors.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 13

Name of the test method	Ishikawa cell test
Clinical endpoint	Adverse effects on implantation.
Cell (line)	Ishikawa cells.
Method description	The test aims to identify chemicals which alter the expression of embryo-implantation-associated target genes in human endometrial adenocarcinoma Ishikawa cells. Ishikawa cells are cultured to subconfluency and incubated for 0.5 to 24 hours with test substances. mRNA levels of target genes are analysed by quantitative RT-PCR (qPCR). Purpose: Screening test to predict detrimental effects of chemicals on the human endometrium.
SOP	Yes.
Endpoints	mRNA levels of progesterone receptor and other implantation-related target genes.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 14

Name of the test method	Trophoblast cell test
Clinical endpoint	Adverse effects on implantation.
Cell (line)	Trophoblast lines BeWo and HTR-8Svneo.
Method description	Cells of the trophoblast lines BeWo and HTR-8Svneo are taken as *in vitro* surrogates to evaluate toxic effects of chemicals that might occur during placental development. BeWo and HTR-8Svneo cells are seeded in 96-well plates and treated with different concentrations of chemicals or vehicle controls. After 24h of incubation, cell viability is assayed and supernatants are analysed for hormone secretion. Purpose: Screening test to identify chemicals that could potential interfere with the physiology of human placenta.
SOP	Yes.
Endpoints	(1) trophoblast cell viability (2) hormone secretion (β-hCG).
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 15

Name of the test method	Human placental pericytes
Clinical endpoint	Adverse effects on implantation.
Cell (line)	Human pericytes isolated from human placenta.
Method description	Placental pericytes (PP) are undifferentiated cells that support placental blood vessels. The screening test aims to identify chemicals affecting proliferation and differentiation of human PP. PP are isolated, cultured and exposed to test chemicals. Treated cells are labelled with antibodies for growth factor receptors and a marker for cell proliferation.
SOP	Yes.
Endpoints	Number of cell cycles, differentiation state.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 16

Name of the test method	Human endometrial endothelial cells
Clinical endpoint	Adverse effects on implantation.
Cell (line)	Primary human endometrial endothelial cells (hEECs).
Method description	Primary hEECs serve as indicator cells to detect adverse effects of chemicals on endometrial functions such as implantation. HECCs are exposed to chemicals and tested for viability (WST-1assay, ELISA), for proliferation (BrdU assay, ELISA) and for changes in expression of a spectrum of endothelial cell markers (qRT-PCR).
SOP	Yes.
Endpoints	Cell viability, cell proliferation and changes in expression of HIF-1α, VEGFR-1/2, PECAM-1/CD31, CD34, vWf, Annexin V, VEGF-A, ER β, PGDH, CYP1A1 and CYP1B1.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 17

Name of the test method	Placental perfusion
Clinical endpoint	Adverse effects on embryo development.
Cell (line)	–
Method description	Human placental perfusion investigates transplacental transfer (indicating foetal exposure) and metabolism of test chemicals. Placental gene expression as a control of placenta functionality is determined in parallel. For this purpose a recirculating system, where the maternal and foetal sides of the placenta are perfused separately, is used, closely resembling physiological conditions.
SOP	Yes.
Endpoints	Transport of test substances between maternal and foetal blood compared to that of a positive control (antipyrine).
How is a positive/negative result defined? / Applicability / Negative control / Performance	
R&D stage	
Positive control	Antipyrine.
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability assessment is in progress.

Table 18

Name of the test method	Embryonic stem cell test (EST)
Clinical endpoint	Adverse effects on embryo development.
Cell (line)	Mouse embryonic stem (ES) cells (D3) and mouse fibroblast (BALB/3T3); rat hepatocytes.
Method description	The EST uses two permanent mouse cell lines (1) ES cells to represent undifferentiated (embryonic) tissue and (2) 3T3 fibroblast cells to represent differentiated (adult) tissue. Upon cytokine (LIF) removal, ES cells form multi-cellular aggregates (embryoid bodies, EBs) consisting of derivatives of all three embryonic germ layers. Plating of the EBs allows further differentiation of cells into beating cardiomyocytes. Treatment with the test chemical may affect myocardial differentiation of ES cells (analysed by light microscopy) or be cytotoxic to ES or 3T3 cells (measured in parallel with the MTT assay). Inhibition of differentiation and cytotoxicity determined in ES and 3T3 cells, respectively, are selected endpoints to predict the embryotoxic potential of test chemicals. So far only directly toxic chemicals could be detected in the EST. The *in vitro* test was therefore combined with a metabolic activation system to allow prediction of embryotoxic effects of chemicals that require metabolic activation. Freshly isolated rat hepatocytes are incubated with test chemicals. Supernatants containing active metabolites are added to D3 ES cells which are then differentiated under the standard EST protocol.
SOP	Yes.
Endpoints	ID50 (50% inhibition of ES cell differentiation), IC50 ES (50% inhibition of ES cell growth), IC50 3T3 (50% inhibition of 3T3 cell growth).
How is a positive result defined?	Change in ES cell differentiation.
How is a positive result expressed?	The substance is classified as weak or strong embryotoxic.
Applicability	Not yet available.
Positive control	5-Fluorouracil.
Negative control	–
Performance	Validated test.
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive, drug development.
Which R would the test method impact?	Reduction.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Improvements of the validated EST have been explored in a recent ReProTect/ECVAM workshop.

Table 19

Name of the test method	WEC assay
Clinical endpoint	Adverse effects on embryo development.
Cell (line)	–
Method description	In the whole embryo culture system (WEC) the developing embryo is analysed during the critical phases of organogenesis outside the maternal animal. Gestation day 10-12 rat embryos are cultured during organogenesis *in vitro* and treated with test chemicals. After 48h, exposure-related effects are studied using growth and developmental changes as readout. In ReProTect a metabolism-competent variant of the assay has been investigated. Test substances are pre-incubated with rat liver microsomes for bioactivation and metabolites are added to the WEC system. The system is limited to the identification of pro-teratogens that are activated by phase-I enzymes of drug metabolism.
SOP	Yes.
Endpoints	Growth and morphology of embryos.
How is a positive/negative result defined? / Applicability / Positive/negative control / Performance	
R&D stage	
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive, drug development.
How can the test be used?	As a stand-alone test or combined in a testing strategy.

Table 20

Name of the test method	MELN
Clinical endpoint	Reproductive toxicity.
Cell (line)	Human breast cancer cells (MCF-7).
Method description	MELN cells are oestrogen-sensitive human breast cancer cells (MCF-7) which are stably transfected with a luciferase reporter plasmid controlled by repeats of oestrogen responsive elements (EREs). Changes in luciferase activity are indicative of ER activation or inhibition which may be mediated by a tested chemical. Purpose: Screening of chemicals for mode of action on the human ER (agonism or antagonism).
SOP	Yes.
Endpoints	Luciferase activity (EC50) and LDH leakage.
How is a positive/negative result defined? / Negative control / Performance	
Not yet available	
Applicability	Detection of oestrogenic or anti-oestrogenic activity.
Positive control	E2 (agonist), 4-OH-Tamoxifen (antagonist).
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive, drug development.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability assessment is in progress.

Table 21

Name of the test method	ER-CALUX
Clinical endpoint	Reproductive toxicity.
Cell (line)	Human U2-OS cells stably transfected.
Method description	ER-CALUX cells are human U2-OS cells stably transfected with a gene encoding the human oestrogen receptor α (ERα) and a luciferase reporter plasmid controlled by repeats of oestrogen responsive elements (EREs). Ligand-activated ERα induces the reporter gene luciferase. Purpose: Identification of chemicals that produce estrogenic effects.
SOP	Yes.
Endpoints	Luciferase activity in cellular lysates.
How is a positive/negative result defined? / Positive/negative control / Performance	
R&D stage	
Applicability	Detection of estrogenic activity.
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive, drug development.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability assessment is in progress.

Table 22

Name of the test method	AR-CALUX
Clinical endpoint	Reproductive toxicity.
Cell (line)	Human U2-OS cells stably transfected.
Method description	AR-CALUX cells are human U2-OS cells stably transfected with a gene encoding the human androgen receptor α (AR) and a luciferase reporter plasmid controlled by repeats of androgen responsive elements (AREs). Ligand-activated AR induces the reporter gene luciferase. For further information see ER-Calux® assay. Purpose: Identification of chemicals that produce androgenic effects.
SOP	Yes.
Endpoints	Luciferase activity in cellular lysates.
How is a positive/negative result defined? / Positive/negative control / Performance	
R&D stage	
Applicability	Detection of androgenic activity.
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive, drug development.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability assessment is in progress.

Table 23

Name of the test method	ER-binding assay
Clinical endpoint	Reproductive toxicity.
Cell (line)	–
Method description	Potential affinity of chemicals for the oestrogen receptor-α (ER) is evaluated by means of an ER binding assay using recombinant human ERα. Radiometric binding assay: the ligand [H-3]-estradiol and the human recombinant full length ER are incubated in the presence/absence of test compound. Unbound ligand and unbound test compound are absorbed by charcoal. The ligand bound to the ER is measured by liquid scintillation counting. Purpose: Screening and priorisation of compounds according to their affinity to the ER.
SOP	Yes.
Endpoints	Binding of radiolabelled ligand to the ER.
How is a positive/negative result defined? / Positive/negative control / Performance	
R&D stage	
Applicability	Detection of ER binding activity.
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive, drug development.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability assessment is ongoing.

Table 24

Name of the test method	AR-binding assay
Clinical endpoint	Reproductive toxicity
Cell (line)	–
Method description	Potential affinity of chemicals for the androgen receptor (AR) is evaluated by means of an AR binding assay using recombinant rat receptor. Radiometric binding assay: The ligand [H-3]-Methyltrienolone (R1881) and the receptor, a rat re-combinant fusion protein to thioredoxin, containing both hinge region and ligand binding domain of the rat AR, are incubated in the presence/absence of test compound. Unbound ligand and test compound are absorbed by charcoal. The AR-bound ligand is measured by liquid scintillation counting. Purpose: Screening and priorisation of compounds according to their affinity to the AR.
SOP	Yes.
Endpoints	Binding of radiolabelled ligand to the AR.
How is a positive/negative result defined? / Positive/negative control / Performance	
R&D stage	
Applicability	Detection of AR-binding activity.
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive, drug development.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability assessment is in progress.

Table 25

Name of the test method	PALM assay
Clinical endpoint	Reproductive toxicity.
Cell (line)	PC-3 human prostate adenocarcinoma.
Method description	PALM cells are human prostate adenocarcinoma cells. The system is used to identify chemicals that stimulate or antagonise androgen receptor (AR)-mediated transactivation as evaluated by means of a cellular transactivation assay. Purpose: Screening and priorisation of compounds that activate or antagonize the AR.
SOP	Yes.
Endpoints	Luciferase activity in cellular lysates and LDH activity in medium.
How is a positive/negative result defined? / Positive/negative control / Performance	
R&D stage	
Applicability	Detection of androgenic or anti-androgenic activity.
Can the test method be used in a regulatory safety context?	Yes. REACH, Cosmetics Directive, drug development.
How can the test be used?	As a stand-alone test or combined in a testing strategy.
Other important remarks?	Transferability and intra-laboratory variability assessment is in progress.

Next steps

Even though ReProTect allowed the identification of new valuable *in vitro* assays that are capable of mimicking special parts of the mammalian reproductive cycle, more information is required prior to their regulatory acceptance. Preliminary information regarding reproducibility and predictivity of the various tests is now available through the project. The next step consists of a thorough evaluation of the newly developed tests for entering into a formal validation exercise. In this context, a complete dossier will be compiled for each assay in order to collect the relevant information required for judgment. At the same time, manuscripts will be prepared by the project partners and jointly submitted for publication in a 'Special Issue' of Reproductive Toxicology, already mentioned above, which will then give a comprehensive overview on the scientific achievements that have been obtained in the ReProTect project. In addition, it is planned to include a paper in this issue that gives detailed information on the chemicals selected for

ReProTect and a 'Position paper' that will discuss the issue of how the newly developed tests might become part of a regulatory framework.

Due to the complexity of the mammalian reproductive cycle, it cannot be expected that the presently available alternative *in vitro* assays are able to replace current animal testing for reproductive toxicity of drugs and chemicals. The test systems deliver, however, mechanistic information that is very valuable for prioritization, particularly during early drug development (search for the least toxic) or in REACH (search for the most toxic), and are therefore well suited to lead to a reduction in animal use when combined with the animal test. Furthermore, sound mechanistic information will help to clarify equivocal results and/or issues relevant to hazard identification/characterisation, such as species-specificity, whether reproductive/developmental effects depend on unspecific general toxicity, identification of most sensitive – 'critical' – targets, etc.: the availability of an appropriate *in vitro* testing battery will, therefore, both avoid many unnecessary additional animal tests and support a science-based, as well as cost- and time-effective, toxicological assessment.

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VITROCELLOMICS

Reducing animal experimentation in preclinical predictive drug testing by human hepatic *in vitro* models derived from embryonic stem cells

Contract number:	LSHB-CT-2006-018940
Project type:	Specific Targeted Research Project (FP6)
EC contribution:	€ 2 942 000
Starting date:	1 January 2006
Duration:	42 months

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

Objectives

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

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 fourth to sixth leading cause of death in hospitalised patients in the USA. 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 are 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 *in vitro* tools available. 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 reliably could predict human metabolism and disposition would markedly reduce the need for animal experimentation for this purpose.

The overall objectives of the VITROCELLOMICS project are:

- 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 hindering weak lead candidates from entering clinical phases with innovative human hepatic cell systems;
- delivering an *in vitro* testing system with adjacent methodology pertinent for validation in GLP/SOPs environment for absorption, metabolism, and toxicity;
- to markedly reduce the use of animals in drug testing, refine the model system under consideration and to replace the animals currently used. This is the ultimate aim.

The clinical expertise in the project is 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. The coordinator has managerial background from an international pharmaceutical company as well as the project leader from one of the SME partners. Three partners represent a solid reputed bioengineering background. Other important partners are one SME focused on development of standardised assay conditions for drug testing, and the European Centre for Validation of Alternative Methods (ECVAM). Links to animal care institutes are also added to the project.

Experimental Design

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

The experimental design of the project involves improvement of culturing procedures for directing hESC differentiation towards a mature hepatocyte phenotype and expansion and handling of the derived hepatocytes. This work also includes identification of hepatocyte markers and a thorough characterisation of hepatocyte phenotype of cells after undergoing the new differentiation protocols.

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

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

Established assays for toxicity are evaluated with reference hepatocytes for comparisons with the hESC-derived hepatocytes and new biosensor technologies are evaluated for use in toxicity screening. The comparative studies of hepatocytes derived from hESC with established *in vitro* models and hepatocyte reference cell types are 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 pre-validation is multi-wavelength fluorescence spectroscopy (Fritzsche et al., 2009).

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

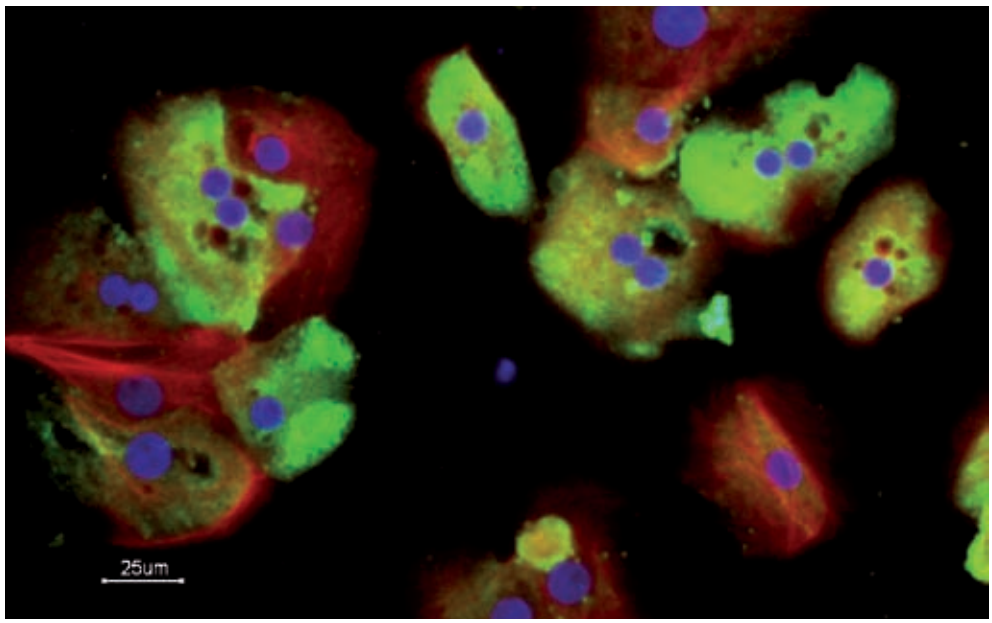
Name of the test method	Induction of Cytochrome P450 enzymes in HepaRG cell line applying an 'n-in-one approach'
Clinical endpoint	Induction of CYP1A2, CYP2B6, CYP2C9, and CYP3A4.
Cell (line)	HepaRG.
Method description	The human hepatoma cell line HepaRG displays hepatocyte-like functions and functionally 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, an n-in-one approach will be followed. After exposure to the test compound at six 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: β-naphthoflavone, CYP2B6, CYP2C9, CYP3A4 rifampicin.

Name of the test method	Induction of Cytochrome P450 enzymes in HepaRG cell line applying an 'n-in-one approach'
Negative control	CYP1A2rifampicin: CYP2B6, CYP2C9, CYP3A4 β-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.

Results

The protocols used to direct differentiation of hESC lines towards mature hepatic phenotype have been continuously modified, refined and tested. A milestone result of the project is 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).

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.



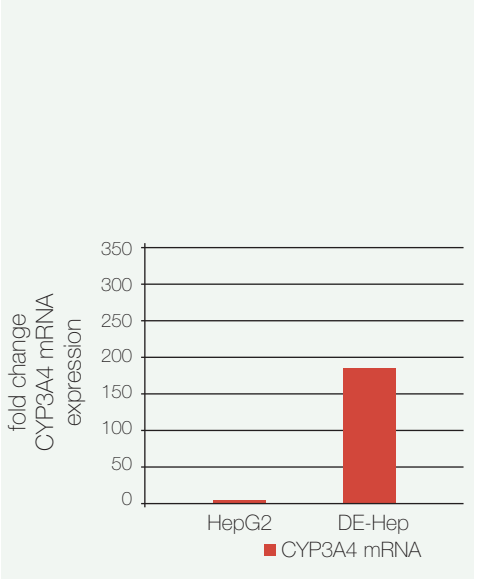


Figure 2. QPCR data for mRNA-expression of CYP3A4 for human cell line HepG2 and hESC-derived hepatocyte–like cells (DE-Hep). Expression level in HepG2 is set to 1.

Generation of toxicity and drug metabolism data have been completed with the selected test compounds using assays evaluated and selected earlier in the project. Assays used have been optimised for hepatocyte reference cells in the partner laboratories. It was concluded that established toxicity assays are rather poor predictors of clinical toxicity and that hESC-derived cells may have much higher potential for desired level of predictability. Furthermore, it is 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).

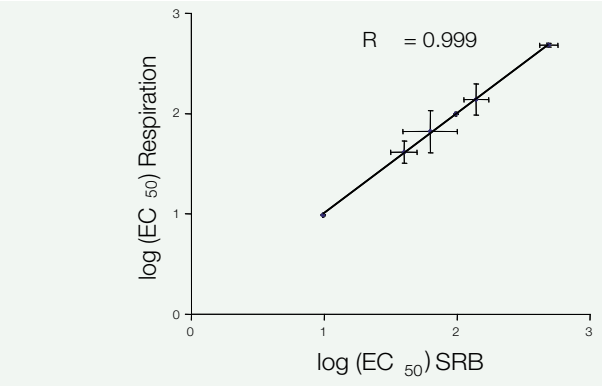


Figure 3. Comparison of EC50 values obtained in respiration assay versus those obtained in SRB (protein) assay showing an excellent correlation.

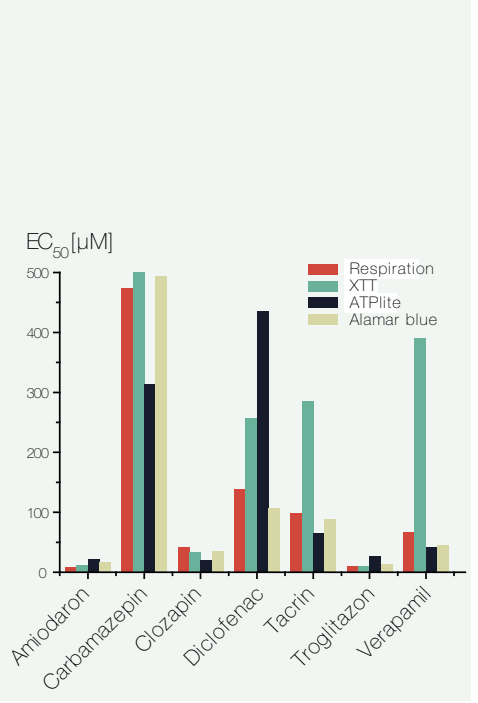


Figure 4. Comparison of EC50 values obtained in various cytotoxicity assays and on-line dynamic respiration assay.

The partners working on test method development are using 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.

Preliminary respiration measurements using hESC-derived hepatocytes, cryopreserved primary hepatocytes and Hep G2 in 24 well respiration assays have been carried out. Diclofenac and amiodarone have been tested in a range of concentrations for the determination of the EC50 values.

The task of establishing models for metabolome studies for detection of toxic effects using hepatocyte reference cells and test compounds indicate that drug-induced effects can be detected at sub-toxic levels compared to established cytotoxicity assays.

Metabolite balancing and 13C labelling studies have been carried out. Using metabolite balancing a flux map was established for Hep G2 cells (Figure 5). To obtain further information, labelling studies with 13C labelled glucose and glutamine were carried out and the isotopomer fractions were calculated (Figure 6).

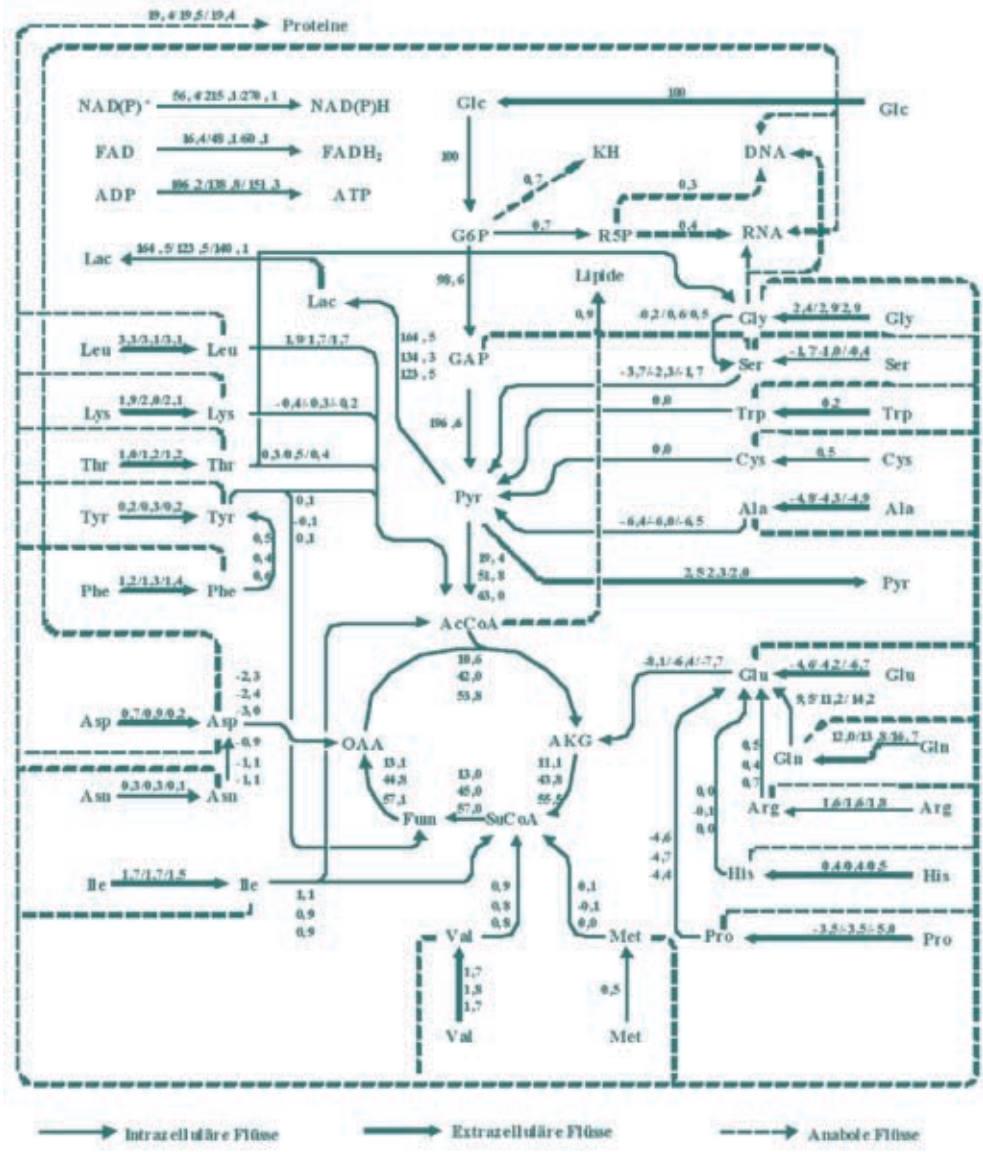


Figure 5. Flux map for Hep G2 treated with 10µM and 31.6µM diclofenac for 50 hours. The top/left value represents untreated control.

Cultivation conditions in small-scale bioreactors have been further evaluated and adapted using primary hepatocytes. The optimisation made has resulted in markedly higher survival rates when hepatocytes were cultured as 3D structures (Figure 7). Also, improved maintenance of hepatocyte functionality in three-week cultures was obtained, showing more that 10-fold higher in UGT activity and 3-fold higher ECOD activity (Miranda et al., 2009). Furthermore, optimisation of 3D bioreactor cultivation for the purpose of large-scale expansion of cells is in progress, at present,

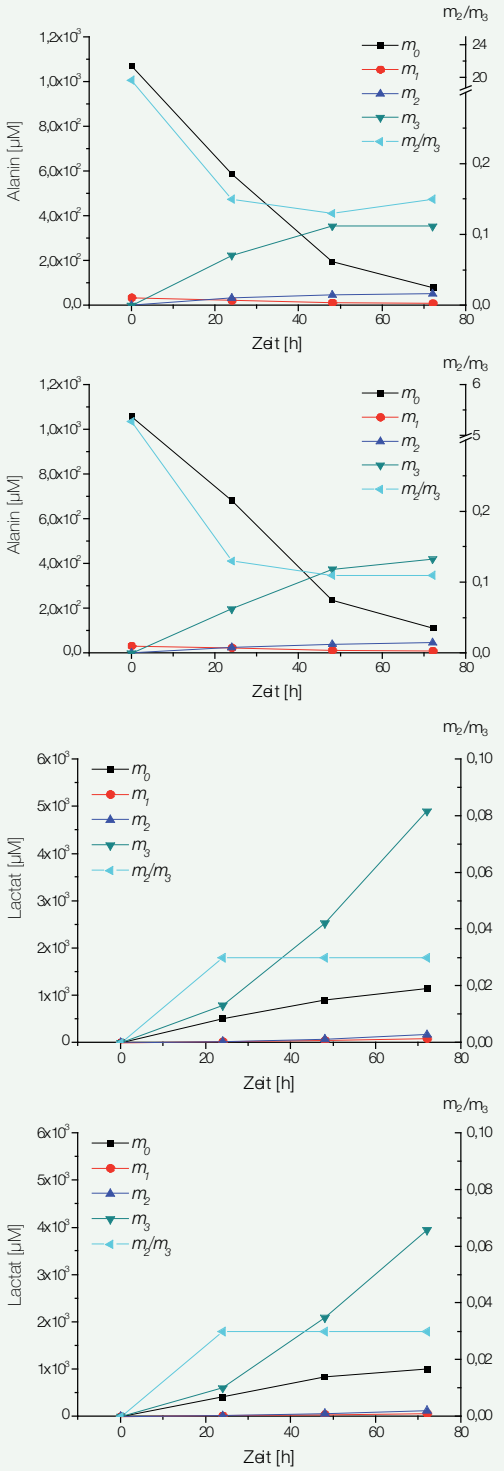


Figure 6. Changes in mass isotopomer concentrations upon exposure to tacrine (50µM) using [U-13C] glucose as substrate. The left figures indicate untreated control.

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 8). Optimisation studies of the system have been conducted in the project with various hepatic cells and cell lines under varying conditions which will be presented in near future (Zeilinger et al.). Basic culture media optimisation has been an integral part of the studies (Dong et al., 2008).

Figure 7. 3D bioreactor with fully controlled conditions.

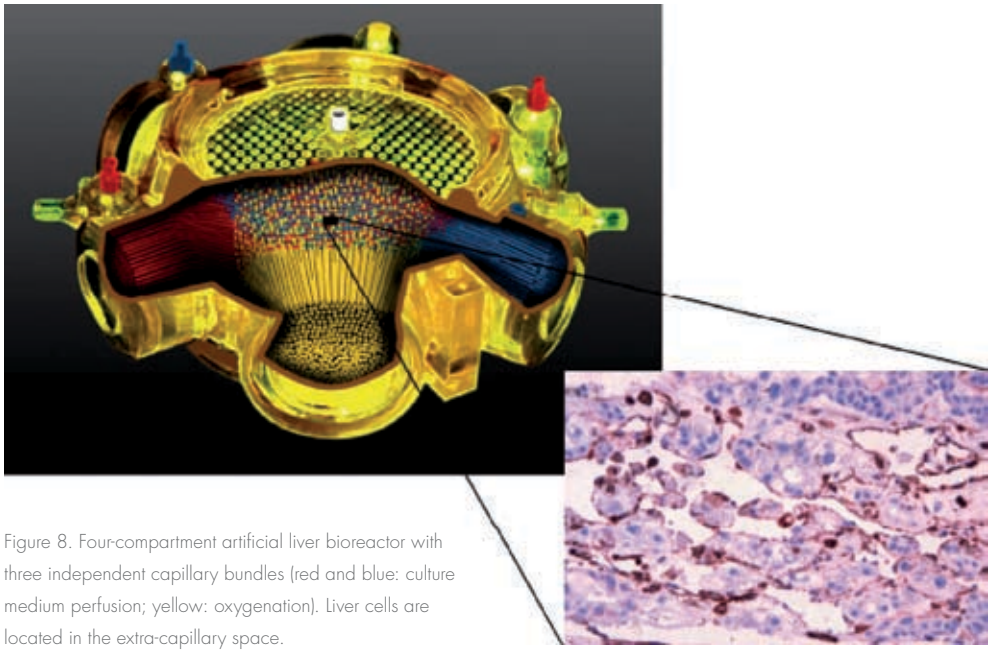


Figure 8. Four-compartment artificial liver bioreactor with three independent capillary bundles (red and blue: culture medium perfusion; yellow: oxygenation). Liver cells are located in the extra-capillary space.

A method on metabolic liver enzyme (CYP) induction is pre-validated using HepaRG reference cells provided by BioPredic (see Table 1). The structure of the pre-validation study is based of the modular approach proposed by ECVAM (Hartung et al., 2004). The study design was discussed and verified during a Stakeholder meeting at Ispra hosted by ECVAM. The pre-validation study is performed by Pharmacelsus (lead laboratory), IBET and ECVAM (naïve laboratories).

The pre-validation contains four phases:

1. In the **first phase**, within-batch reproducibility is tested.

2. In the **second phase**, between-batch and within-laboratory reproducibility is evaluated.

3. In the **third phase**, between-laboratory reproducibility is 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 to Phase 1 and 2 was performed by Pharmacelsus. Functional enzyme activity assays were performed subsequent to 48 hours of exposure to chemical inducers, performed by one or two operators at the lead laboratory. Naïve laboratories perform a transferability training assay in order to initiate Phase 3 of the pre-validation study.

Furthermore, partners are establishing SOPs for selected assays, guided by partner ECVAM providing the consortium with expertise on test method validation.

Next steps

For the last part of the project, the focus is on delivery of hESC-derived hepatocytes to partners working with development of assays for metabolism and toxicity and on the partners working with 3D bioreactor development. A priority is the completion of the CYP-induction pre-validation study.

The results of Phase 1 and Phase 2 of the CYP induction pre-validation study are currently under analysis by ECVAM. The naïve laboratories perform a transferability training assay in order to initiate Phase 3 of the pre-validation study. Phase 3 will start as soon as the results of Phase 1 and 2 are evaluated and the transferability training for the naïve laboratories has been conducted. For Phase 3, four (blinded) test items will be tested by the three laboratories on three different HepaRG batches.

Respiration assay on hESC-derived hepatocytes will be carried out using diclofenac and amiodarone in a range of concentrations for 48 hours. The experiment will also include human primary hepatocytes and Hep G2 cells as reference cell lines. In addition, labelling experiments with [U-13C] glucose, [1,2 -13C] glucose and [U-13C] glutamine will be carried out in parallel using sub-toxic concentrations of diclofenac and amiodarone.

Publications

1. Simone Beckers, Fozia Noor, Ursula Müller-Vieira, Manuela Mayer, Alexander Strigun, Elmar Heinzle. 'High throughput, non invasive and dynamic toxicity screening on adherent cells using respiratory measurements' (submitted).

2. Marc Lübberstedt, Ursula Müller-Vieira, Manuela Mayer, Klaus M. Biemel, Fanny Knöspel, Daniel Knobeloch, Andreas K. Nüssler, Jörg C. Gerlach, Katrin Zeilinger. 'Metabolic activity and clearence capacitiy of the hepatic cell line HepaRG compared with primary human hepatocytes' (submitted).

3. Jia Dong, Carl-Fredrik Mandenius, Marc Lübberstedt, Thomas Urbaniak, Andreas K. N. Nüssler, Daniel Knobeloch, Jörg C. Gerlach, Katrin Zeilinger. 2008. 'Evaluation and optimization of hepatocyte culture media factors by design of experiments (DoE) methodology'. Cytotechnology 27, 251 – 261.

4. Miranda JP, Leite SB, Muller-Vieira U, Carrondo MJT, Alves PM. 2009. 'Towards extended functional hepatocyte *in vitro* culture'. Tissue Engineering Part C, 15 (in press).

5. Miranda J, Carrondo MJT, Alves P. 2007. 3D Cultures: Effect on the hepatocytes functionality. Noll (ed.) 'Cells & Culture', Springer (in press).

6. Fozia Noor, Jens Niklas, Ursula Müller-Vieira and Elmar Heinzle (2009). 'An integrated approach to improved toxicity prediction for the safety assessment during preclinical drug development using Hep G2 cells'. Toxicology and Applied Pharmacology (in press).

7. Simone Beckers, Fozia Noor, Ursula Müller-Vieira, Manuela Mayer, Alexander Strigun and Elmar Heinzle (2009). 'High throughput non invasive dynamic toxicity screening on adherent cells using respiratory measurements'. Toxicology in-vitro (in press).

8. Michael Fritzsche, J. Magnus Fredriksson, Maria Carlsson, Carl-Fredrik Mandenius, 2009. 'A cell-based sensor system for toxicity testing using multi-wavelength fluorescence spectroscopy'. Analytical Biochemistry 387, 271–275.

9. Ek M, Söderdahl T, Küppers-Munther B, Edsbagge J, Andersson TB, Björquist P, Cotgreave I, Jernström B, Ingelman-Sundberg M, Johansson I. 'Expression of Drug Metabolizing Enzymes in Hepatocyte-like Cells Derived from Human Embryonic Stem Cells'. Biochem. Pharmacol. 74 (2007) 496-503.

10. Ursula Mueller-Vieira, Manuela Mayer, Jennifer Nussbaum, Lorenz Maahs, Klaus M. Biemel; 14th Congress on Alternatives to Animal Testing – Linz 2007 & 11th Annual Meeting of MEGAT – Middle European Society for Alternative Methods to Animal Testing; September 28-30, 2007, Linz, Austria - Poster presentation: Smart *in vitro* test sequence for 'hepato-profiling' of potential new drugs.

11. PharmaForum-SW, November 12, 2007, Mainz, Germany - Poster presentation: Pharmacelsus GmbH; Research & Development: EU-Project 6th Framework: VITROCELLOMICS.

Patent applications filed / Exploitable results

1. Integration of a Cytochrome P450 induction assay on primary human hepatocytes to the Pharmacelsus portfolio.

2. Integration of the (pre-validated) method for Cytochrome P450 induction on the HepaRG cell line to the Pharmacelsus portfolio.

3. A novel population of hepatocytes derived via definitive endoderm (DE-hep) from human blastocysts derived stem cells. Patent filed July 2007 – US60/935,003 by Cellartis AB.

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MEMTRANS

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

memtrans

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

Objectives

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. 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. Specifically, we wish to identify their influence on concentration-time profiles of xenobiotics in the circulation.

The first objective of the study is the characterisation and assessment of the functionality and biological characteristics of each *in vitro* model as well as the characterisation of the functional MDR status of the cell lines.

These studies were developed according to previously determined parameters and written common Standard Operation Procedures (SOP's) for cell culture, transport experiments and transference among laboratories using the cell lines Caco-2, MDCKII and MDCKII-MDR. This pre-validation study of the selected cell lines will enable the further evaluation of the culturing methods and permeation experiment protocols along with the interlab and intralab reproducibility of the results. The validation of the cell lines will lead to the better correlation of the results between the laboratories in the future.

Experimental design

Name of the test method	*In vitro* model of intestinal absorption			
Clinical endpoint	Oral route of administration, drug-transporter, drug-drug and drug-food interactions.			
Cell (line)	Caco-2, MDCKII-WT, MDCKII-MDR.			
Method description	Different cell cultures with varying levels of secretion carriers were tested with a group of model drugs. The assays were developed with common standard operation procedures to diminish the interlaboratory variability and to permit a better comparison of the models performance. The *in vitro* permeability values of model drugs were compared/correlated with the available literature data of pharmacokinetic parameters and bioavailability in humans to check the predictability of each culture model and to characterise the systems parameters. The *in vitro* permeability was determined by growing the cells monolayers in Transwell™ chambers. The solution of the drug at different concentration was added to either apical or basolateral side while free medium is added to the opposite side. The amount of drug appearing in the acceptor chamber was measured by liquid chromatography. The permeability value is obtained from the slope of the linear regression of amount of drug in acceptor chamber versus time, divided by initial concentration in donor and area of transport. The permeability values obtained at different concentrations were used to obtain Km (Michaelis-Menten constant) and Vm (Maximal velocity of transport) parameters by non-linear regression of a combined passive and Michaelis-Menten equation.			
SOP	Yes.			
Endpoints	Develop reliable *in vitro* models with improved predictability of *in vivo* biopharmaceutical characteristics.			
How is a positive result defined?	Cell monolayer integrity	Electrical measurement	Trans-Epithelial Electrical resistance (TEER) measurement, depending on the filter area, can reveal toxicity or an opening of tight junctions induced by the drug.	
		Permeability measurement of test compounds	Mannitol, Lucifer Yellow.	Low paracellular permeability.
			Metoprolol.	High transcellular permeability.
	P-glycoprotein (P-gp) expression	Western blotting.		
		Rhodamine 123 permeability.		
		Calcein Assay.		

Name of the test method	*In vitro* model of intestinal absorption
How is a positive result expressed?	Absolute values.
Applicability	Compounds tested: Celiprolol, Fexofenadine, Quinidine, Loperamide, Talinolol, Saquinavir, Paclitaxel.
Positive control	Demonstration of low paracellular permeability with either ^{14}C-Mannitol or Lucifer Yellow. Demonstration of high transcellular permeability with Metoprolol or ^{3}H-Metoprolol. Demonstration of Pgp efflux using Rhodamine 123.
Negative control	Permeability value in the absence of monolayer.
Performance	Statistical differences between High-Low permeability values of High-Low permeability markers.
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Reduce and Replace *in vivo* testing, Refining and improving *in vitro* methodologies.
How can the test be used?	As a part of an alternative/integrated testing strategy.

Results

The following tables and figures show the experiments done in Caco-2, MDCKII and MDCKII-MDR cells.

CACO-2 CELLS

Demonstration of low paracellular permeability

Experiments to demonstrate the appropriateness of Caco-2 cells for the low paracellular permeability were done using 14C-Mannitol in ACB and Lucifer yellow (LY) in UVEG and SOLVO. The experiments were conducted in the apical to basolateral (ab) direction and in the absence and presence of shaking (orbital shaker set at 100 r.p.m.).

Table 1. Comparison of the results of the demonstration of the appropriateness of monolayers for the low paracellular permeability gathered in different laboratories. SD denotes standard deviation; RSD denotes relative standard deviation.

Caco-2 cells								
Partner	Compound	Passage Number	n	Day	Mode	Papp x 10^{-6} [cm/s]		
						Ab	SD	RSD%
UVEG	LY	37	6	14	shaking	0.28	0.21	62,89
		38	6	14	no-shaking	0.11	0.05	47.95
SOLVO	LY	24	3	14	shaking	0.51	0.32	62.75
		24	3	14	no-shaking	0.42	0.25	59.52
ACB	^{14}C-Mannitol	43	3	25	shaking	1.89	0.25	13.23
		44	3	25	no shaking	1.36	0.1	7.35

Demonstration of high transcellular permeability with Metoprolol

Experiments to demonstrate the appropriateness of Caco-2 cells for the high transcellular permeability were done using Metoprolol. The experiments were conducted in the apical to basolateral (ab) direction and in the absence and presence of shaking.

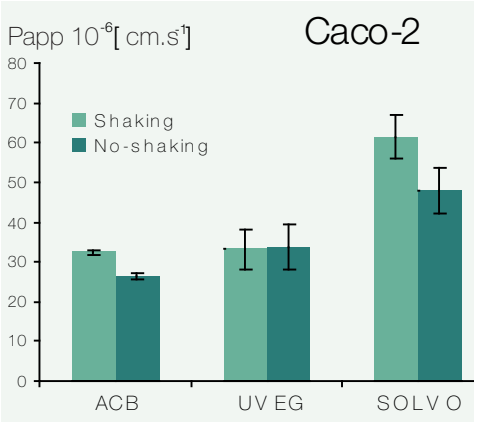


Figure 1. Comparison of the results of the demonstration of the appropriateness of monolayers for the high transcellular permeability gathered in different laboratories (n=3-6).

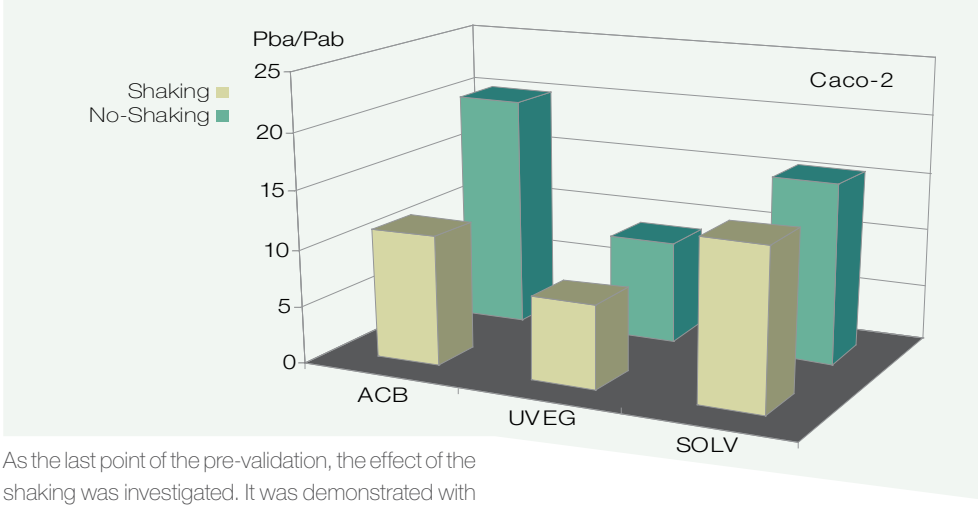
Demonstration of Pgp efflux with Rhodamine 123

Experiments to demonstrate the presence of the Pgp efflux were done using Rhodamine 123. The experiments were conducted in the apical to basolateral (ab) direction, basolateral to apical (ba) direction and in the absence and presence of shaking.

The ability of the Caco-2 cell monolayers for the separation of low and high permeability were demonstrated using Mannitol, Lucifer yellow and Metoprolol. The pre-validation method was able to determine the Pgp expression of the Caco-2 cells. In the experiments that were conducted with a selective Pgp substrate Rhodamine 123 it was demonstrated that efflux ratios that were determined by all the partners were higher than seven in any case.

Table 2. Comparison of the results of the demonstration of Pgp efflux with Rhodamine 123 gathered in different laboratories.

Caco-2 cells										
Partner	Passage Number	n	Day	Mode	Papp x 10^{-6} [cm/s]					
					ab	SD	RSD%	ba	SD	RSD%
UVEG	37	3	14	shaking	1.40	0.30	21.03	10.08	2.13	21.39
	38	3	14	no-shaking	1.33	0.10	7.69	11.92	1.67	14.25
SOLVO	24	3	14	shaking	1.28	0.02	1.56	17.9	1.23	6.87
	24	3	14	no-shaking	0.93	0.23	24.73	14.6	2.1	14.38
ACB	43	3	25	shaking	0.51	0.04	7.84	5.77	0.6	10.40
	44	3	24	no shaking	0.22	0.01	4.55	4.42	0.51	11.54



As the last point of the pre-validation, the effect of the shaking was investigated. It was demonstrated with the above results that shaking can be used without any sacrifice from the monolayer properties.

Figure 2. Comparison of the efflux ratios of Rhodamine 123 in Caco-2 gathered in different laboratories.

MDCKII and MDCKII-MDR CELLS

Pre-validation of the MDCKII and MDCKII-MDR cells were carried out by the partners UVEG and ACB. The quality control markers used and permeability experiment conditions were the same as for Caco-2 cell line validation.

Calcein AM assay was carried out by SOLVO for the determination of the Pgp activity of both cell lines.

Demonstration of low paracellular permeability

Table 3. Comparison of the results of the demonstration of the appropriateness of monolayers for the low paracellular permeability gathered in different laboratories.

MDCKII cells								
Partner	Compound	Passage Number	n	Day	Mode	Papp x 10^{-6} [cm/s]		
						Ab	SD	RSD%
UVEG	LY	51	6	4	shaking	0.25	0.07	26.74
		58	6	4	no-shaking	0.21	0.05	26.63
ACB	^{14}C-Mannitol	12	3	4	shaking	0.88	0.13	14.55
		16	3	4	no shaking	2.32	0.51	21.95

Table 4. Comparison of the results of the demonstration of the appropriateness of monolayers for the low paracellular permeability gathered in different laboratories.

MDCKII-MDR cells								
Partner	Compound	Passage Number	n	Day	Mode	Papp x 10^{-6} [cm/s]		
						Ab	SD	RSD%
UVEG	LY	56	6	3	shaking	0.32	0.04	13.21
		57	6	3	no-shaking	0.63	0.19	30.56
ACB	^{14}C-Mannitol	12	3	4	shaking	1.19	0.12	9.94
		16	3	4	no shaking	2.28	0.23	10.15

Demonstration of high transcellular permeability with Metoprolol

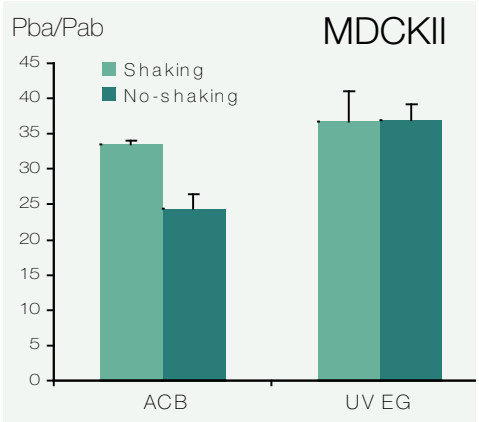


Figure 3. Comparison of the results of the demonstration of the appropriateness of monolayers for the high transcellular permeability gathered in different laboratories (n=3).

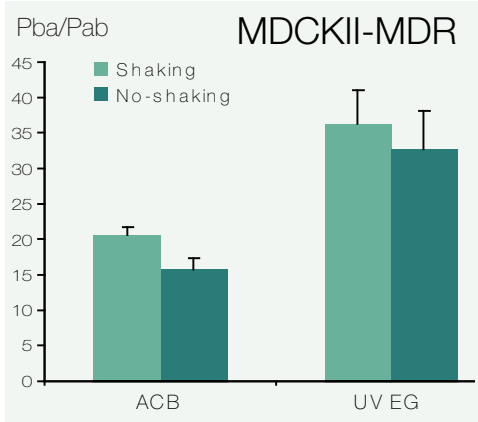


Figure 4. Comparison of the results of the demonstration of the appropriateness of monolayers for the high transcellular permeability gathered in different laboratories (n=3).

Demonstration of Pgp efflux with Rhodamine 123

Table 5. Comparison of the results of the demonstration of Pgp efflux with Rhodamine 123 gathered in different laboratories.

MDCKII cells										
Partner	Passage Number	n	Day	Mode	Papp x 10^{-6} [cm/s]					
					ab	SD	RSD%	ba	SD	RSD%
wUVEG	51	3	4	shaking	2.83	0.61	21.00	10.17	2.84	28.00
	52	3	3	no-shaking	1.41	0.27	19.00	8.19	0.49	6.00
ACB	12	3	4	shaking	0.75	0.09	12.00	3.06	0.43	14.05
	16	3	4	no shaking	0.46	0.11	23.91	1.53	0.19	12.42

Table 6. Comparison of the results of the demonstration of Pgp efflux with Rhodamine 123 gathered in different laboratories.

MDCKII-MDR cells										
Partner	Passage Number	n	Day	Mode	Papp x 10^{-6} [cm/s]					
					ab	SD	RSD%	ba	SD	RSD%
UVEG	56	3	4	shaking	1.88	0.36	19.00	12.22	0.88	7.00
	57	3	3	no-shaking	2.32	0.99	42.00	11.69	0.69	6.00
ACB	12	3	4	shaking	0.65	0.13	20.00	3.48	0.32	9.19
	16	3	4	no shaking	0.35	0.04	11.42	2.26	0.30	13.27

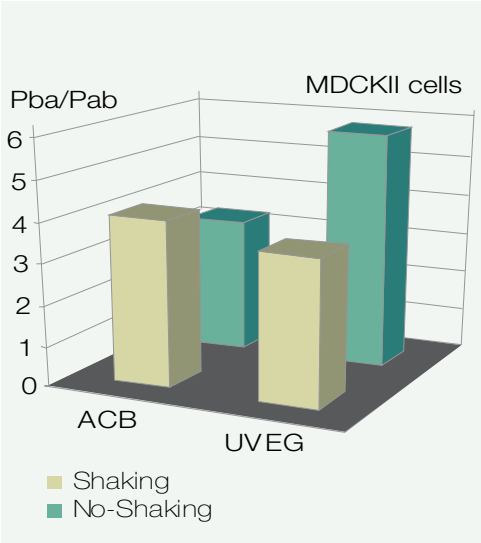


Figure 5. Comparison of the efflux ratios of Rhodamine 123 in MDCKII cells gathered in different laboratories.

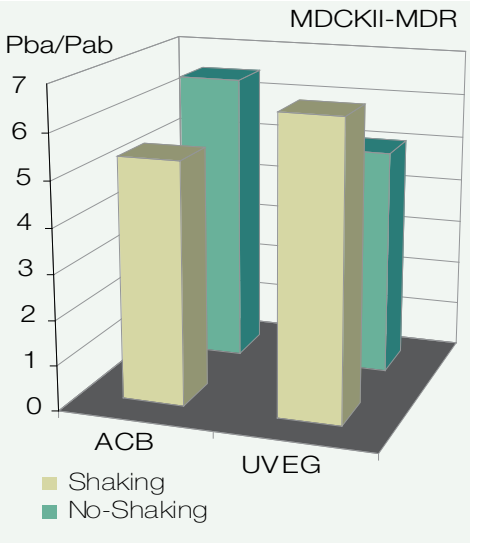


Figure 6. Comparison of the efflux ratios of Rhodamine 123 in MDCKII-MDR cells gathered in different laboratories.

Calcein AM assay

The functions of the multidrug resistance protein (Pgp) were assessed by measuring the accumulation of a fluorescent dye, Calcein, in tumour cells e.g. by flow cytometry. The quantitative MDR1 activity factors (MAFMDR) are calculated from the Calcein AM extrusion assay by using efficient inhibitors of the multidrug resistance protein. This relatively simple and rapid *in vitro* functional assay provides a reliable quantitative measure for cellular multidrug resistance and the activity of the MDR1 protein.

Calcein AM assay was carried in the presence and absence of the inhibitors Verapamil and LY335979 to determine the different Pgp activity between MDCKII and MDCKII-MDR cells.

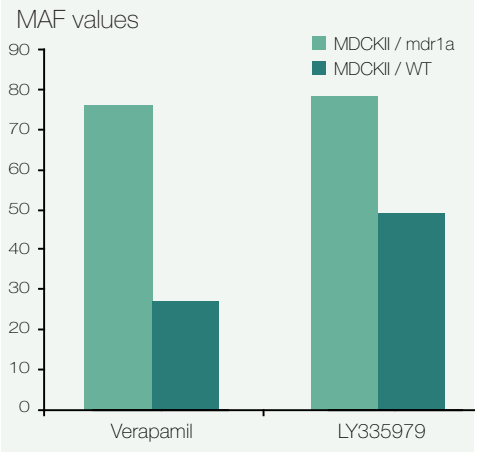


Figure 7. MAF values of the MDCKII/WT and MDCKII/mdr1a cell found using Calcein AM assay.

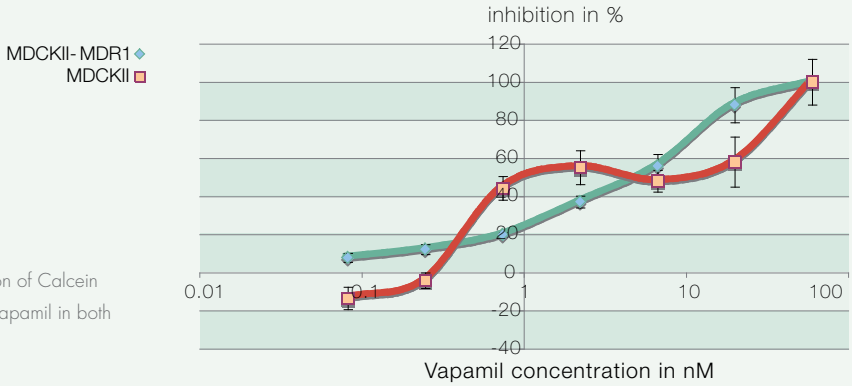


Figure 8. Inhibition of Calcein AM efflux by Verapamil in both cell types.

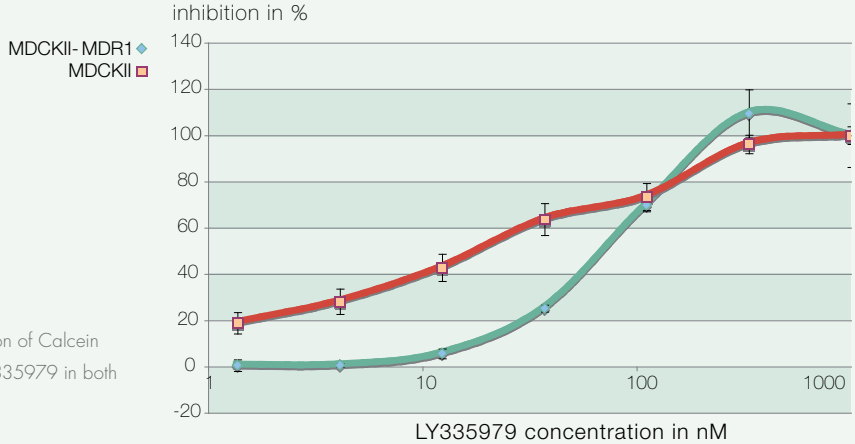


Figure 9. Inhibition of Calcein AM efflux by LY335979 in both cell types.

Conclusion

The ability of the Caco-2 cell monolayers for the separation of low and high permeability were demonstrated using Mannitol, Lucifer yellow and Metoprolol. The pre-validation method was able to determine the Pgp expression of the Caco-2 cells. In the experiments that were conducted with a selective Pgp substrate Rhodamine 123 it was demonstrated that efflux ratios that were determined by all the partners were higher than seven in any case.

The ability of the MDCKII and MDCKII-MDR cell monolayers for the separation of low and high permeability were demonstrated using Mannitol, Lucifer yellow and Metoprolol. In the experiments conducted with Rhodamine, minor differences were observed between the MDCKII and MDCKII-MDR cells in terms of efflux ratios which is further proved with Calcein AM assay.

MDCKII cells express a transporter that transports Calcein. The transport process can be inhibited by LY335979 and Verapamil. MDCKII-MDR1 cells show higher rate of Calcein AM transport. This transport could be also inhibited by LY335979 and Verapamil, but with different inhibition profiles and IC50 values.

These results show that the MDCKII parental cell line expresses a (most probably canine) transporter that has biochemical characteristics similar to human MDR1 (Calcein AM transporter can be inhibited by LY335979 or Verapamil). The MDCKII-MDR1 cells express the human MDR1 transporter resulting in higher MAF values and different inhibition profiles and IC50 values for the two compounds.

As the last point of the pre-validation the effect of the shaking was investigated. It was demonstrated with the above results that shaking can be used without any sacrifice from the monolayer properties.

Finally, the results obtained by different laboratories were comparable with each other which will enable further correlations and the analyses of the repeatability of the further experiments using previously selected substances.

Next steps

Permeability studies have been conducted in the three cell lines at four different concentrations and in the absence and presence of 1 uM zosuquidar (P-gp inhibitor).

Project ongoing tasks

From the data generated the transport parameters of each drug will be characterised in each cell line. The correlation analysis and generation of prediction models includes the analysis of the relationships between physicochemical characteristics and affinity for the carrier and the analysis of the correlation between *in vitro* and *in vivo* results. The biophysical models for drug transport (transport location, binding site, apical-basolateral resistances) will be characterised in each *in vitro* model by using a computer modelling and simulation approach. That will allow obtaining the transport parameters for the correlation with physicochemical variables which eventually could be also included as model co-variates.

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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: LHSB-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.eu/exera

Objectives

Develop novel *in vitro* 2D and 3D models of mouse tissues for the Pharmaco-Toxicological analysis of Estrogen Receptors Interacting Compounds (ER-ICs): liver, skin, bone (non reproductive systems), ovaries and testis (sex-specific reproductive systems) (see Review dedicated to the project on: Genes and Nutrition, Penza et al. 2009; in press).

Experimental design

Description of the methodology used for the generation of the cell systems

The steps to reach the objectives involve several complementary techniques: cell isolation, constitutive and conditional immortalisation, cell banking, 3D-cultures, whole genome expression profiles, *in vivo* imaging, and application of 3D-cultures devices (RCCS Technology):

▸ Cell isolation from tissues of oestrogen reporter mice

Reliable protocols have been established by the partnership and have been applied to the isolation of cells from liver, skin, bone, testis and ovaries of oestrogen reporter mice. Cell cultures with 'physiological' oestrogen-dependent phenotypes have been established for immortalisation. Cell cultures are constantly controlled and characterised with respect to the *in vivo* situation with specific markers (luciferase enzymatic assay, endogenous markers of tissue health and differentiation) and by gene expression profiles.

▶ Immortalisation

This step has been performed by transfection methodologies in 2D and 3D and using suitable constitutive and inducible vectors (tet on/tet off elements under the control of the antibiotic doxycycline). For conditionally immortalised cells, the immortalising gene is switched on for cell production, and off for characterisation and testing. Markers of differentiation have been studied under both conditions. The reversed phenotype expresses tissue specific genes at much higher levels compared to the (on) situation where the immortalising gene (SV40 TA) is expressed and the cells are actively proliferating.

▶ Constitution of a cell bank

Immortalised cell cultures that are satisfying the desired parameters have been expanded and controlled for banking. Selected clones have already been banked for some tissues (skin fibroblasts and mesenchimal stem cells from bone marrow).

3D-cultures adapted to grow cells with an unaltered oestrogen-dependent phenotype

Cell-cell and extracellular matrix-cell interactions play a fundamental role in maintaining the function of numerous organ systems. Hence, tissue engineering represents a good way to overcome limits of monolayer cultures and to maintain tissue-like architecture and functionality. Thus 3D cultures represent a potential bridge to cover the gap between animal models and human studies (Mazzoleni et al. 2009). This project addresses the significance and the potential of 3D *in vitro* systems to improve the predictive value of cell-based assays for safety and risk assessment studies and of new drug development and testing.

▶ Assessment

Assessment of the newly developed cell lines and the 3D-culture systems for the pharmaco-toxicological characterisation of ER-ICs.

Description of the methodology used for the in vivo *analysis*

Modern imaging techniques applied to animal engineering give novel opportunities to create innovative model systems enabling the quantification of, spatio-temporally, the receptor activity on synthetic reporter genes with non-invasive technologies (Di Lorenzo et al. 2008). This molecular imaging makes it possible, for the first time, to measure the activity of a given compound in all the organs of a living mammal without distress or prior sacrifice of the laboratory animal itself. These models are of particular relevance for the study of transcription factors such as NRs, because genes encoding reporter proteins under the control of hormone-NR responsive promoters can be integrated into the genome of small laboratory animals. In these transgenic models, the presence of ligands for specific NRs can be easily detected (Penza et al. 2007; Montani et al. 2008). This *in vivo* analysis fulfils two of the 3Rs' (Refine and Reduce).

Measurable endpoints

In vitro

- Establishment of immortalised cells with physiological functions which are closer to primary cells;
- Establishment of immortalised cells with functional oestrogen-dependent phenotypes and protocols for 2D and 3D-cultures;
- Optimisation of the RCCS Technology for 3D-cultures of oestrogen responsive cells;
- Comparison of data obtained from 2D and 3D-cultures (functional data and genome expression profiling).

In vivo

- *In vivo* expression of the reporter luciferase gene and *in vivo-in vitro* comparisons;
- Data on the characterisation of selected ER-ICs.

Name of the test method	*In vitro* 1 - FIB. 2 - LIV. 3 - BONE. 4 - SERT. 5 – GRAN. *In vivo* The ERE-tK-LUC mouse (detection of chronic exposures by optical imaging)
Clinical endpoint	Hormonal response (oestrogen-dependent gene expression).
In vitro Cell lines	1 - FIB. Immortalised mouse skin fibroblasts 2 - LIV. Immortalised liver cells 3 - BONE. Immortalised osteoclasts 4 - SERT. Immortalised Sertoli cells 5 - GRAN. Immortalised granulosa cells.
In vivo Reporter mice	The ERE-tK-LUC mouse.
Methods description	*In vitro* Cells are grown in either 2D or 3D and exposed to defined compounds (physiological, pharmaceuticals, nutritionals, cosmetics and environmental oestrogens). After exposure, the transgenic marker luciferase, under the control of oestrogen responsive sequences, is measured and quantified. Its value provides an estimate of the potency and cell specificity of the test compound. *In vivo* After exposures of the reporter mice to the compounds, the transgenic marker luciferase is measured in the whole body and quantified. Its value provides an estimate of the potency and tissue specificity of the test compound and of its ability to interfere with endogenous hormones.
SOP	Yes/No.
Endpoints	Modulation of luciferase and oestrogen target genes.

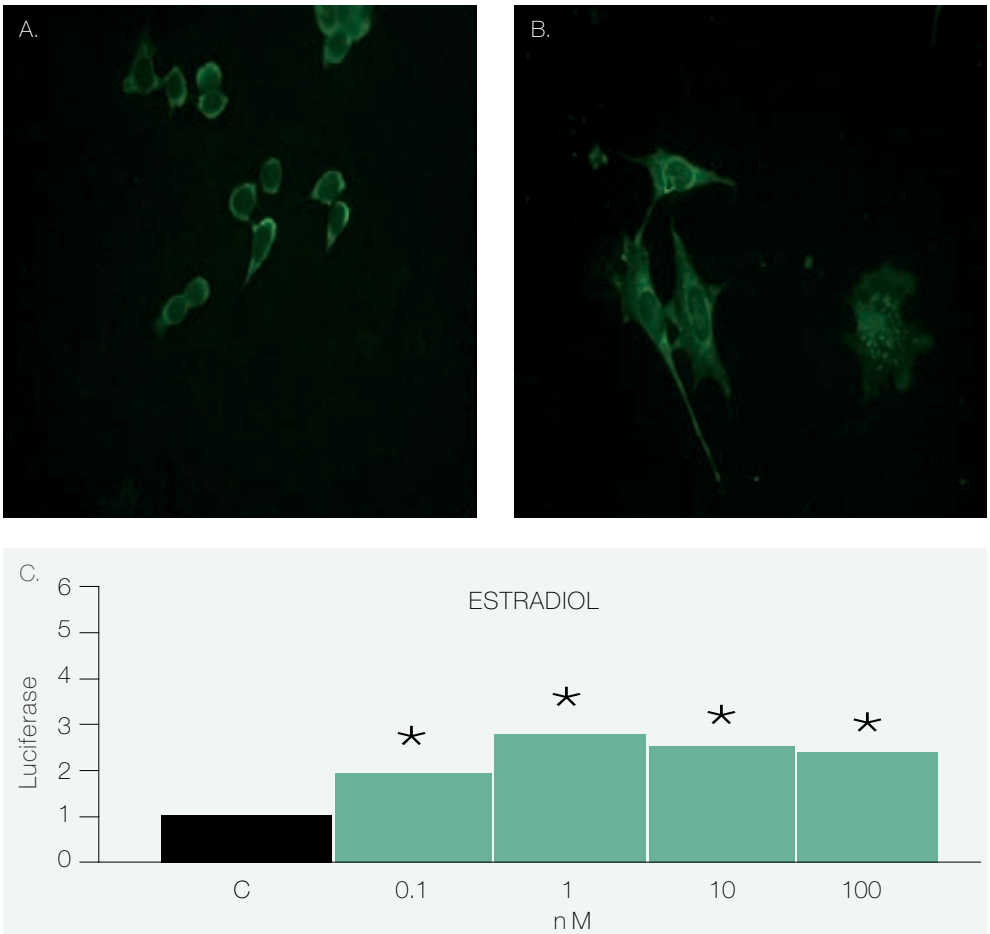
How is a positive result defined?	For both the *in vitro* and the *in vivo* systems, positive results are considered those in which the model is responsive to oestrogens (activation of the reporter gene up to a statistically significant level). Negative results are those in which changes of the reporter expression do not reach statistical significance (unresponsive cells or mice).
How is a positive result expressed?	A positive result can be expressed as both fold change or as absolute value.
Applicability	The test compounds represent classes of hormonally active chemicals: PHYSIOLOGICAL OESTROGENS: Estradiol. DRUGS: Raloxifene, Ospemifene, Lasofoxifene. FOOD COMPOUNDS: Genistein, Daidzein, Resveratrol. ENVIRONMENTAL CHEMICALS: BPA, ßBHC, Cadmium.
Positive control	Estradiol.
Negative control	Vehicle.
Performance	Sensitivity: from Low to High. Specificity: to be determined. Accuracy: good.
Can the test method be used in a regulatory safety context?	Yes, when fully characterised, it can be used to study exposures to hormonally active chemicals. For regulatory requirements: ECVAM.
Which R would the test method impact?	*In vitro* Replacement and Reduce. *In vivo* Reduce, Refine.
How can the test be used?	Both as a stand-alone method and as part of an alternative/integrated testing strategy.
Other important remarks?	The mouse from which these systems have been derived is itself an alternative to the use of mice as in classical pharmaco-toxicology. Thus it contributes to 'Reducing' the number of testing animals and 'Refining' the system.

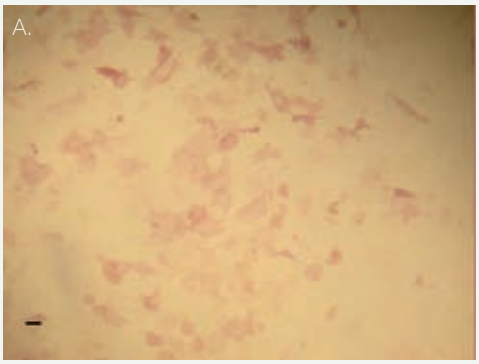
Results

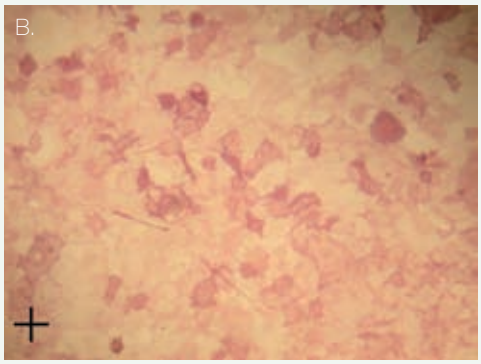
In vitro *(cell systems)*

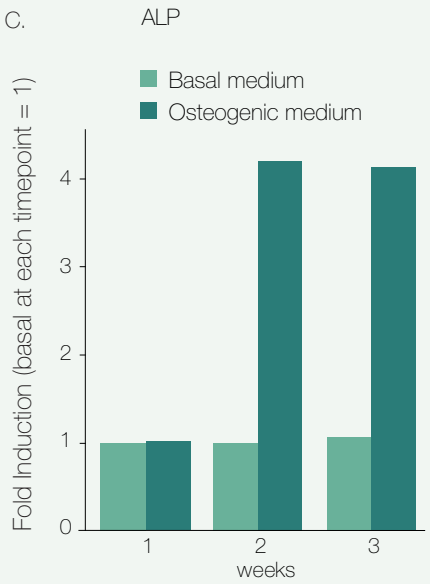
Cells have been isolated from five mouse tissues (skin, liver, bone, testis, ovaries), expanded, and immortalised (constitutive and/or conditional immortalisation) (Figures 1 through 4). Clones have been selected and are tested for maintenance of phenotype and oestrogen responsiveness both in 2D and in 3D cultures. Culture systems are under comparison by whole genome profiling and phenotyping (Figure 7). Preliminary results show that the same cell clones (skin fibroblasts and liver cells) cultured in the RCCS device (3D) have a more differentiated phenotype when compared to cell cultures on Petri dishes (2D) (Figure 6). Whole genome expression profiles indicate that several pathways and genes are up regulated in 3D compared to 2D.

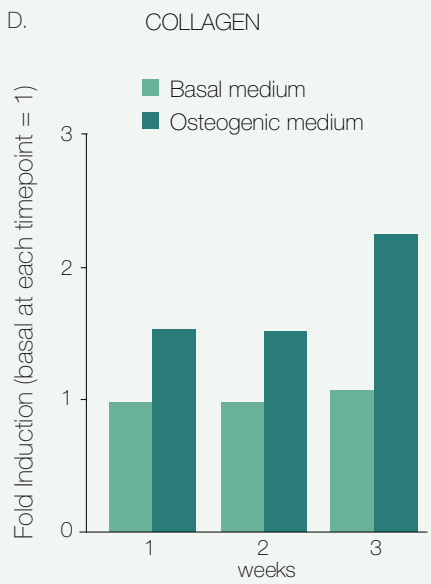
Figure 1. Immortalised skin fibroblasts. A) Cells immortalised through the reversible expression of the TAg oncogene (plus doxycycline). B) Reversed phenotype (minus doxycicline). C) Responsiveness to Estradiol.



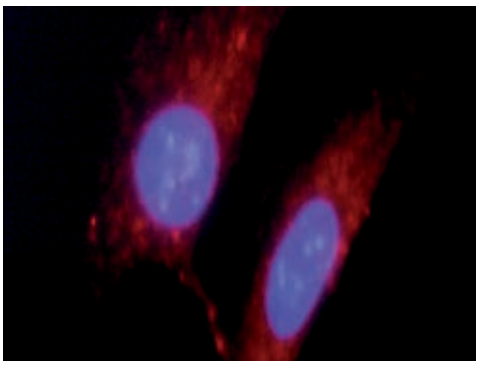


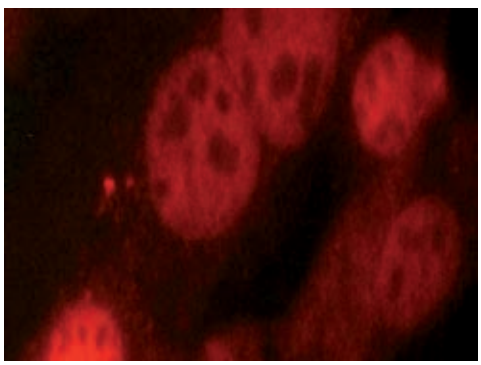




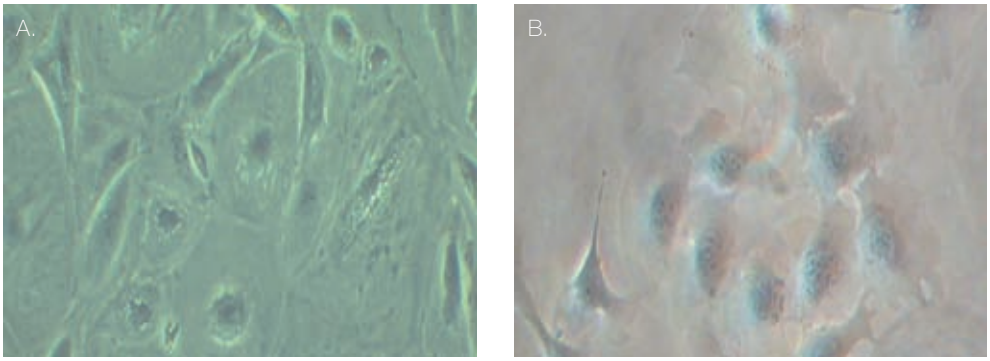


▲ Figure 2. (A and B) Immortalised mesenchimal stem cells and osteogenic differentiation. (C and D) Expression of cell specific markers of osteogenic differentiation.



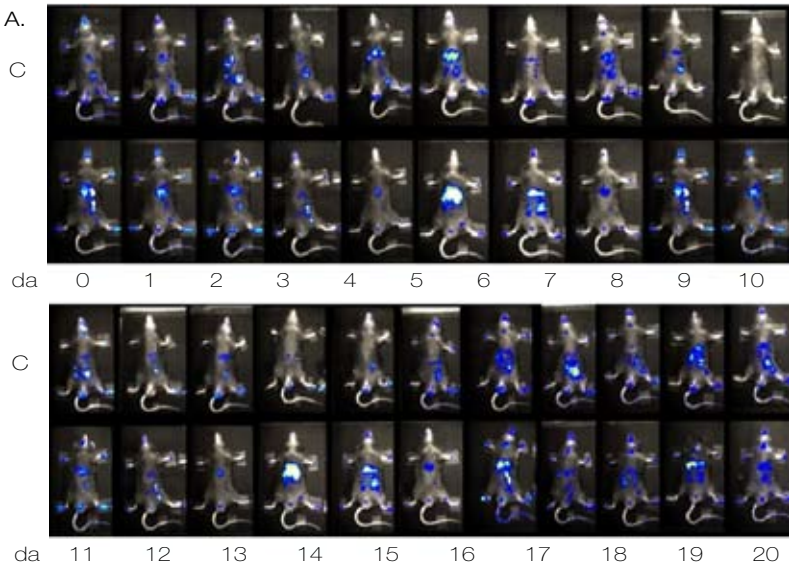


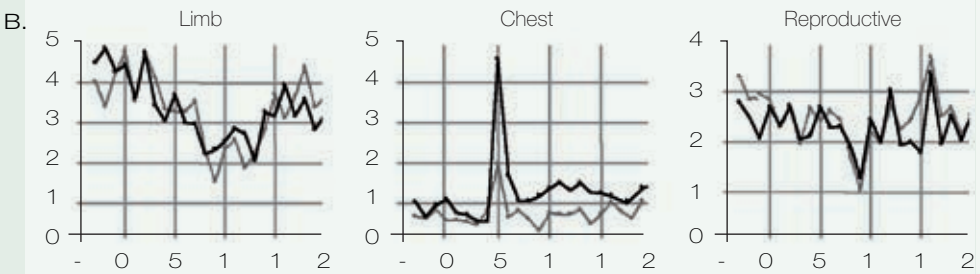
▼ Figure 3. Differentiation markers in immortalised Sertoli cells. Immunostaining of differentiation markers in immortalised Sertoli cells.

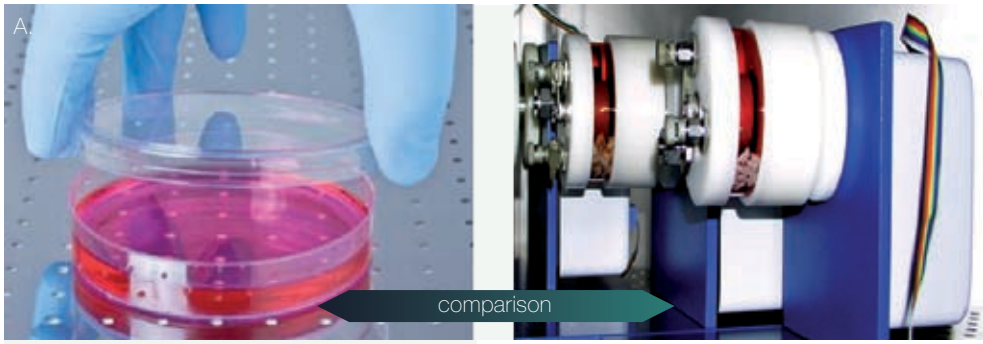


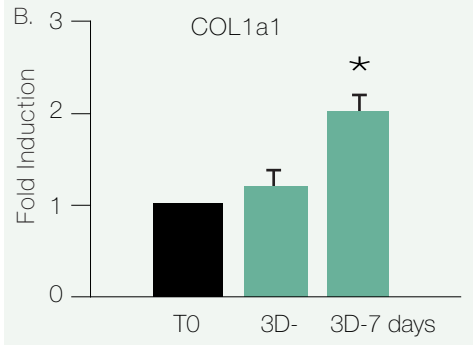
▲ Figure 4. Immortalised granulosa cells. Two clones.

▼ Figure 5. *In vivo* imaging. Chronic exposure to estrogenic chemicals (genistein).





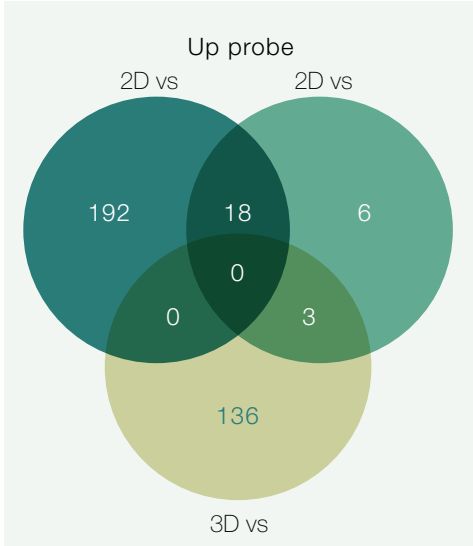


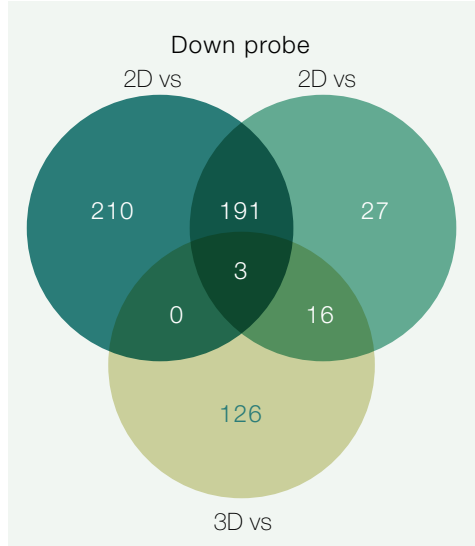


▲ Figure 6. 2D vs 3D culture systems. Expression of skin differentiation markers in 2D vs 3D cultures.

▼ Figure 7. Whole genome expression profiling from immortalised hepatocytes. Venn diagrams. Modulated genes in 2D and 3D culture systems.

Cell clones isolated from the liver and skin have been cultured in 2D and 3D conditions in parallel for seven days from time 0, when the cells were switched to the two systems. Cells were then harvested. RNA was extracted and sent to DV for whole genome expression profiles. The results reveal that in the 3D cultures several cell markers of tissue differentiation, metabolism and health are up regulated. Markers of interest have been selected and their expression confirmed by qReal Time RT-PCR. Genes involved in tissue specific functions show up to 60-fold induction in the 3D culture condition compared to 2D cultures indicating that the RCCS technology allows growing cells and maintaining tissues with more physiological phenotypes. Oestrogen-dependent pathways are under characterisation in 3D-cultures.





Comparison between hormone stimulation in 2D and 3D cultures will be performed by the use of the transgenic marker (luciferase) and by gene expression profiles.

Responsive clones are under testing for oestrogenic chemicals in 2D and 3D. Testing and assessments are proceeding throughout the whole project, until the end. Some selected clones have been quality controlled and banked.

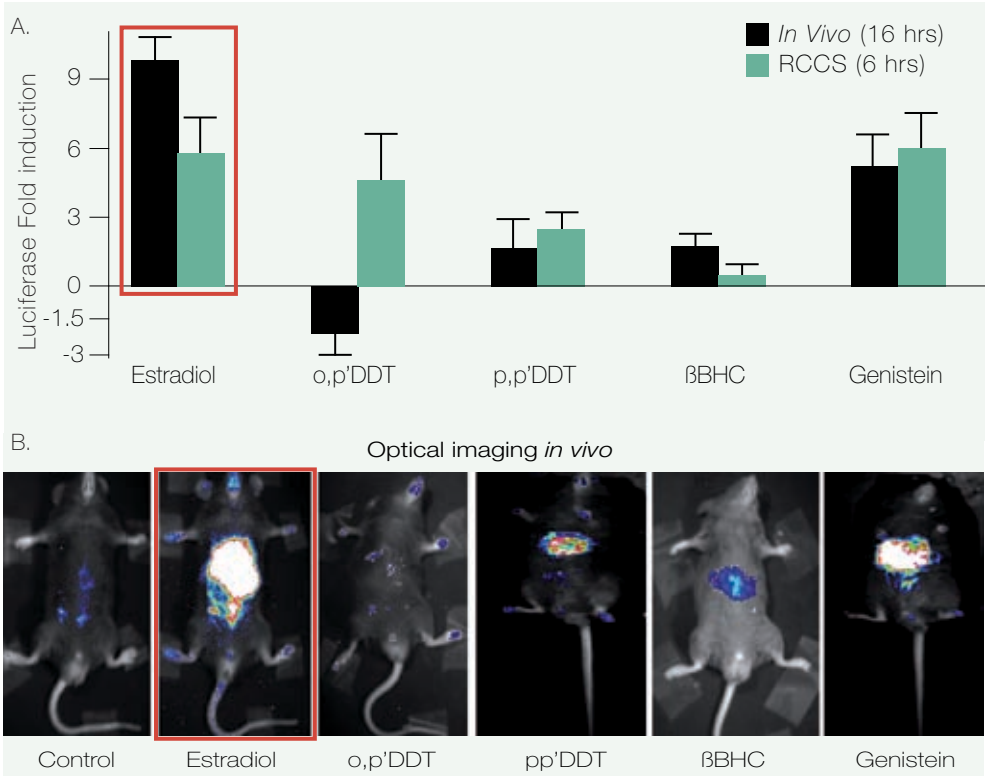
Examples of clones of mouse cells from skin, liver, bone, testis and ovaries, phenotypes and estrogenic response are reported below.

In vivo *(the ERE-tK-LUC mouse model)*

An example of the use of reporter mice for acute and chronic pharmaco-toxicology:

The most interesting feature of the reporter animal systems, is that they are excellent candidates to *Replace* the existing tests that, by their nature, are unable to provide a global view of toxic activity in the whole organism; by means of non-invasive *in vivo* imaging technology, the method provides the opportunity to *Reduce* the number of animals to be used in the *in vivo* tests (Figure 5A and Figure 8). Last but not least, the technology will *Refine* current methods by providing for the first time the possibility to study the effects of oestrogenic chemicals systematically and after long-term exposure (Figure 5B) even to low doses and to mixtures, will nullify the pain for the test animals, and will abolish the necessity of animal sacrifice. The novel technology will be finalised by the end of the project. A new patent is under preparation.

Figure 8. Comparison of *in vivo* and *ex vivo* (liver) (3D) culture systems with respect to the response to estrogenic chemicals (Penza et al. 2006; Steimberg et al. 2009).



Next steps

To define the specificity of the models generated and proceed with the study of oestrogenic compounds *in vitro* and *in vivo*.

Figure 9 shows the project achievements and % accomplishment.

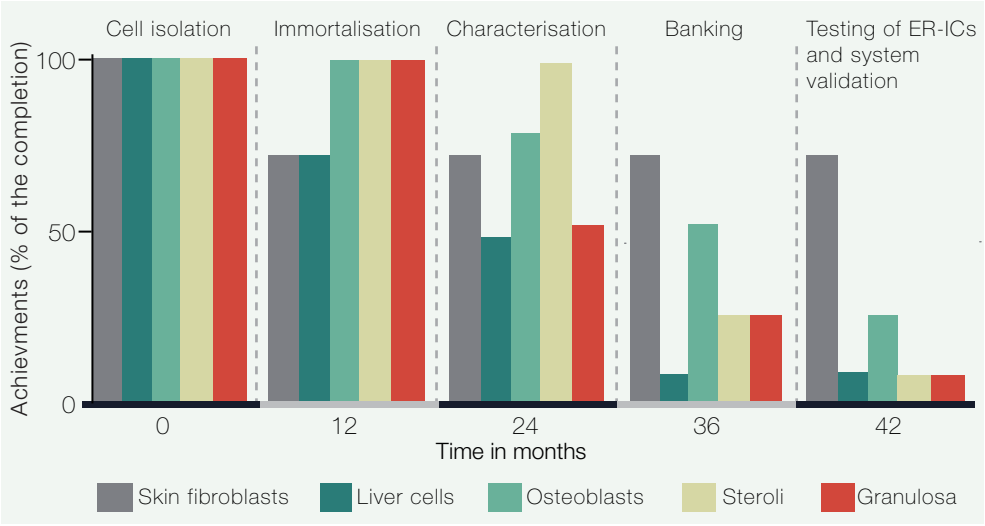


Figure 9. Project achievements and % accomplishments.

Publications

1. Steimberg N, Mazzoleni G, Penza M, Montani C, Maggi A, Ciana P, Caimi L, Di Lorenzo D. Evaluation of Estrogen Receptor Interacting Compounds (ER-ICs) activity in a 3D *ex-vivo* model of mouse liver: comparison with the *in vivo* situation. *Toxicology, under submission.*

2. A CASCADE of Effects of Bisphenol A. M Bondesson, J Jönsson , I Pongratz, N Olea, J Cravedi, D Zalko, H Håkansson, K Halldin, D Di Lorenzo, C Behl, D Manthey, P Balaguer, B Demeneix, J Fini, V Laudet, J-Å Gustafsson. *Journal of Reproduction, in press.*

3. Penza M, Jeremic M, Unkila M, Caimi L, Mazzoleni G, Di Lorenzo D. Searching for alternatives to animal experimentation for hormonal compounds. *Accepted for publication on: Genes and Nutrition.*

4. Di Lorenzo D, Gianpaolo Rando, Paolo Ciana and Maggi A. Development and implementation of new 'in vivo' systems for the characterisation of endocrine disruptors that fulfil the 3Rs principle. *Toxicol Sci. 2008 106(2):304-11.*

5. Mazzoleni G, Di Lorenzo D and Steimberg N. Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? *Genes Nutr. 2009 ;4(1):13-22.*

6. Montani C., Penza M.L., Jeremic M, L., La Sala G., De Felici M., Ciana P., Maggi A, Di Lorenzo D. Whole body estrogenic action of genistein in adults and suckling mice and on fetal testis using estrogen reporter mice. *Toxicol. Sci,* 2008, *103(1):57-67.*

7. M Penza; C Montani; A Romani; P Vignolini; P Ciana; A Maggi; B Pampaloni; L Caimi; D Di Lorenzo. Genistein accumulates in body depots and is mobilized during fasting, reaching estrogenic levels in serum that counter the hormonal actions of estradiol and organochlorines. *Toxicological Sciences 97(2):299-307, 2007.*

8. Penza M, Montani C, Romani A, Vignolini P, Pampaloni B, Tanini A, Brandi ML, Alonso-Magdalena P, Nadal A, Ottobrini L, Parolini O, Bignotti E, Calza S, Maggi A, Grigolato PG, Di Lorenzo D. Genistein affects adipose tissue deposition in a dose-dependent and gender-specific manner. *Endocrinology.* 2006 Dec;147(12):5740-51 *2006.*

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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-2007-037636
Project type: Specific Targeted Research Project (FP6)
EC contribution: € 2 701 611
Starting date: 1 January 2007
Duration: 36 months
Website: http://er-projects.gf.liu.se/~invitroheart

Objectives

The objective of INVITROHEART is to establish stable cell lines that reliably reflect human cardiomyocyte properties by the development of an *in vitro* model that is derived from human embryonic stem cells (hESC). The aim is to deliver a trustworthy and regulatory acceptable *in vitro* model that can be used by pharmaceutical and non-pharmaceutical companies to replace experimental animals in (1) investigations on pharmacological toxicity and safety of compounds in the drug discovery and development processes, and (2) the testing of toxic effects of chemicals according to the new system of the Community 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 toxicity testing of chemical substances replacement of animal testing methods can also be realized in the cosmetics, food, and commodity chemicals industries.

The means to accomplish the objective in INVITROHEART are, 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, (3) a multi-micro-bioreactor platform for high throughput screening of drugs and chemicals. Comparative studies of hESC-CM with established *in vitro* models are carried out in order to validate the new models and methods.

The outcome of INVITROHEART should be a pre-validated *in vitro* model applied to advanced testing methods which will significantly reduce the use of animal experimentation for cardiotoxicity testing. Furthermore, it will strengthen the possibility for the participating SMEs to market new potential products in the areas of *in vitro* assay methods and *in vitro* compound screening. The SMEs' part in this proposal is substantial and the share of the requested budget for the SMEs is 59%.

The clinical expertise in the project is 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 micro-sensor technologies, are the key providers of state-of-the-art technology. The coordinator has managerial background from an international pharmaceutical company as does the project leader from one of the SME partners. Two of the partners represent a solid reputed bioengineering background. Another important partner for the project is the European Centre for Validation of Alternative Methods (ECVAM). Links to animal care institutes are also added to the project.

Experimental Design

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

Advanced *in vitro* methods for assessment of the cardiotoxic potential of drugs and chemicals are evaluated and adapted for use with hESC-derived cardiomyocytes (hESC-CM). Several endpoints are evaluated with focus on electrophysiology parameters (e.g. QT-prolongation) representing functionality as the most critical toxicology parameter. Furthermore, 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 are supplementing the testing platform to enhance the predictability.

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

Results

The most significant achievements during the first half of the project are as follows.

Protocols for generation of cells with characteristics of human cardiomyocytes from hESC have been established and a 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) (Asp et al.);

The hESC-CM have been compared with adult human cardiac tissue and the levels of several cardiac markers are of similar or higher magnitude as compared with the adult tissue;

Functional characterisation of the hESC-CM has been achieved with electrophysiological assessment using MEA technology and cells have been demonstrated to respond to established pharmaceutical blockers of specific ion channels with expected effects;

Development of a prototype electrophysiology sensing technology, including development and optimisation of appropriate software, enabling recording of hESC-CM action field potentials;

Development and optimisation of a prototype optical microsensor technology for oxygen consumption measurements (respiration) and generation of data

demonstrating that this technology is suitable for toxicity testing of pharmaceuticals;

Several cell-based assays with different toxicological endpoints have been thoroughly evaluated. Extensive data generation with test compounds and optimisation of the calculation of toxic effects have been achieved. All partners use test compounds from a defined list and from the same provider;

Several assays have been generated to evaluate toxicological effect of hESC-CM on a single-cell basis;

Test systems for detection of cellular effects at subtoxic concentrations of test compound are being developed based on metabolomics and fluxomics modelling. Promising results have been generated;

Detection methodology for cardiac troponin T has been optimised for surface plasmon resonance technology (Andersson et al. 2009).

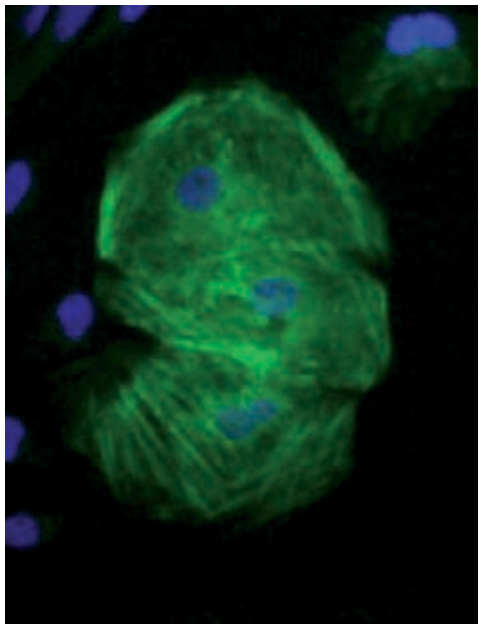


Figure 1. Dissociated hESC-CM stained for the cardiac specific protein troponin, indicating that phenotype is retained when cardiomyocytes are in a single cell format (troponin, green; nuclei, blue; scale bar, 50 µm). This shows potential for use in single cells applications, such as voltage clamp analysis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

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

Spontaneously beating syncytia of cardiac myocytes differentiated from human embryonic stem cells can, by electrophysiological recordings, be used to address parameters relevant for safety pharmacology. These recordings can be performed by non-invasive extracellular electrophysiology, such as the micro-electrode arrays (MEAs) and QT-Screens systems (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 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.

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.
MYH6	Myosin, heavy polypeptide 6, cardiac muscle, alpha (cardiomyopathy, hypertrophic 1).
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.

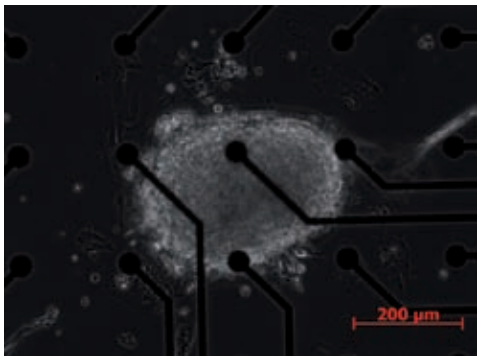


Figure 2. hESC-CM cluster on a multi-electrode array (MEA) plate for field potential recordings. The cells are from partner Cellartis AB and the MEA system is from partner Multi Channel Systems GmbH.

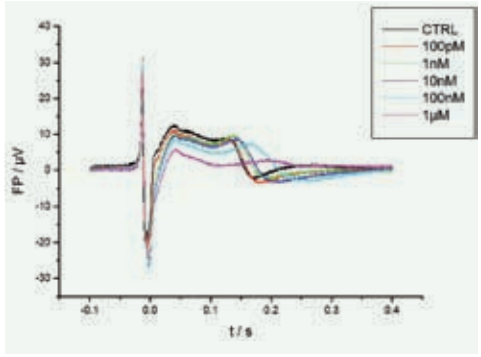


Figure 4. Field potential recordings from hESC-CM with MEA technology showing dose-dependent increase in re-polarization time (QT-prolongation) by the hERG-blocker E-4031.

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.

An aluminum holder for small plastic vessels (from 96-well strip plates) with integrated optical oxygen sensors is tested and evaluated for use with the SensorDish Reader for respiration measurements (Figure 5). Thus, less sample volume is necessary, which significantly reduces the numbers of cells needed for detection of the oxygen consumption. For homogenisation of the oxygen ingress in the 24 wells, a cover clamp system (from Applikon) was modified for use with the SensorDish Reader. The homogeneity of the oxygen ingress into the wells using this system was tested successfully with the sodium sulphite method.

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.

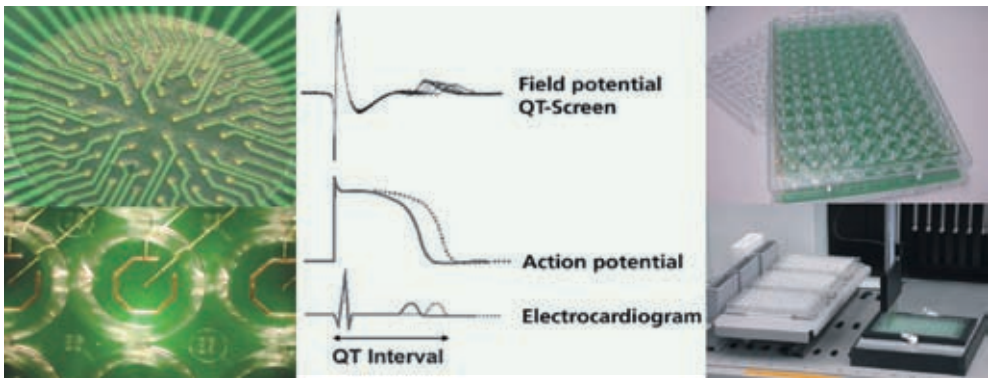


Figure 5. The Sensor Dish Reader (SDR) system from partner PreSens GmbH. This optical fluorescence-based sensor technology allows for optical on-line respiration measurements and monitoring of pH in the cell cultures. Monitoring of the important physiological parameters pH and oxygen is essential for 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.

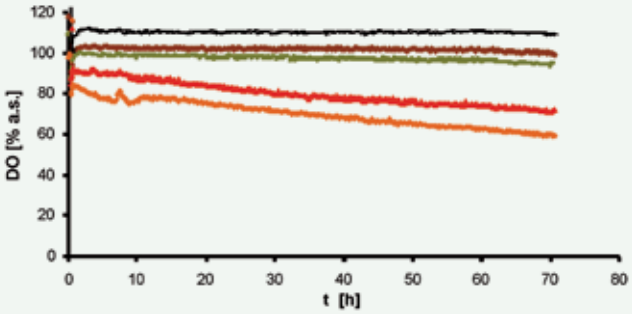


Figure 6. Respiration measurement on 12 hESC-CM clusters/well in a modified format with smaller inlays decreasing the volume to 300µl/well (see Figure 5). Test compound treatment results in a dose-dependent decrease in cell respiration. DO: dissolved oxygen.

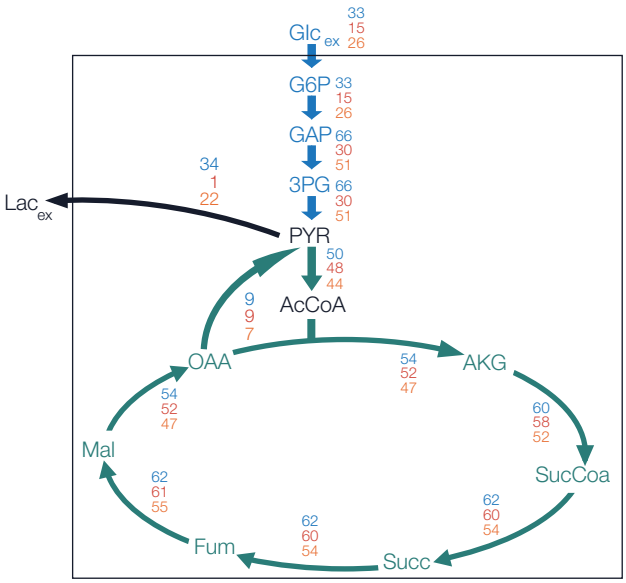
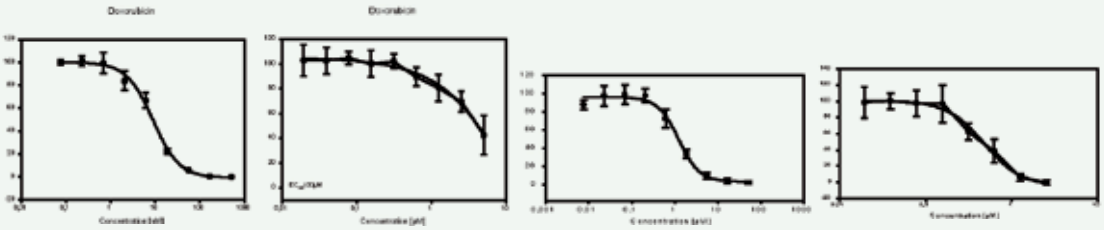


Figure 7. Simplified metabolic network model using metabolite balancing for HL-1 cardiomyocytes. Effects were observed after exposure to sub-toxic concentrations of verapamil for 48 hours. The upper most value: untreated control; middle value: 4µm verapamil; lower value: 4nM verapamil.



Non-cardiomyocyte [THP-1]	Neonatal rat cardiomyocyte	Mouse cardiomyocytes [HL-1]	hESC-cardiomyocytes
EC50 = 0.009 µM	EC50 = 30 µM	EC50 = 1.2 µM	EC50 = 0.5 µM
Effect of doxorubicin on selected cell types			

Figure 8. Effect of test compound after 48h on metabolic activity analysed with Resazurin assay. EC50 values are shown for hESC-CM and reference cell types used in the project.

Next steps

The current production campaign and provision of hESC-CM to project partners has provided a valuable experience in the production of large quantities of these cells and logistics of distribution to user labs. Protocols, originally developed in the R&D lab at Cellartis, have been modified and adapted for GMP manufacture and converted into SOPs and compliance to GLP and quality assurance principles. Frequent feedback from the recipients has allowed for a constant review of the utility of the hESC-CM. We have subsequently made adjustments in hESC-CM format, quantity, size or characteristic to provide material preferable to the individual end-user. For example, we have been able to supply hESC-CM as clusters, in bulk or isolated as individuals, as attached single cells or as monolayers. This work is currently ongoing and is expected to be an important experience of the INVITROHEART project.

Other work on the metabolic characterisation of the hESC-CM is going on. Metabolite balancing and labelling studies with [U-13C] glutamine and [1,2-13C] glucose will be carried out.

In addition to electrophysiological parameters, other biological systems are planned to be evaluated for their potential to predict cardiotoxicity on a cellular as well as sub-cellular level. Factors to be considered in the forthcoming INVITROHEART work that are well known contributors to cardiotoxicity, including metabolic activity, membrane integrity, mitochondrial transmembrane potential, reactive oxygen species, and intracellular calcium flux. For integration of these endpoints into the testing platform, a significant benefit would be if several, if not all, endpoints could be multiplexed into a single system suitable for adaptation to high throughput screening. Furthermore, hESC-CM cell requirement has to be further reduced to allow testing with a limited cell source. This could be accomplished by shifting the system from a 96-well to a 384-well or even 1536-well system.

Cardiac tissues, including the hESC-CM clusters have very high cell density. Dissociation of such tissues has proved difficult where care must be taken to avoid disruption of membrane ion channels. Many platforms require the use of single cells or monolayers, so we are currently optimising the dissociation protocols to facilitate the production of good quality single cells and monolayers.

Publications

1. Asp J, Steel D, Jonsson M, Améen C, Dahlenborg K, Jeppsson A, Lindahl A and Sartipy P. Characteristics of Cardiomyocyte Clusters Derived from Human Embryonic Stem Cells: Effect of *in vitro* maturation and comparison with human heart tissue. (Submitted)

2. Jonsson MKB, van Veen TAB, Goumans MJ, Vos MA, Duker G, and Sartipy P. Improvement of cardiac efficacy and safety models in drug discovery by the use of stem cell derived cardiomyocytes. Expert Opinion on Drug Discovery 2009 4(4):357-372.

3. Steel D, Hyllner J, Sartipy P. Cardiomyocytes derived from human embryonic stem cells - characteristics and utility for drug discovery. Curr Opin Drug Discov Devel. 2009 Jan;12(1):133-40.

4. Synnergren J, Adak S, Englund MC, Giesler TL, Noaksson K, Lindahl A, Nilsson P, Nelson D, Abbot S, Olsson B, Sartipy P. Cardiomyogenic gene expression profiling of differentiating human embryonic stem cells. J Biotechnol. 2008 Mar 20;134(1-2):162-70.

5. Meyer T, Sartipy P, Blind F, Leisgen C, and Guenther E. New Cell Models & Assays in Cardiac Safety Profiling. Expert Opin Drug Metab Toxicol. 2007 Aug;3(4):507-17.

6. Sartipy P, Björquist P, Strehl R, and Hyllner J. The application of human embryonic stem cell technologies to drug discovery. Drug Discovery Today 2007 Sep;12(17-18):688-99. Epub 2007 Aug 30.

7. Björquist P, Sartipy P, Strehl R, and Hyllner J. Human ES cell derived functional cells as tools in drug discovery. Drug Discovery World, Winter 2007/08, Vol. 9, 17-24.

8. Andersson H, Kågedal B, Mandenius CF. Surface plasmon resonance measurement of cardiac troponin T levels in cardiomyocyte cell culture medium as indicator of drug-induced cardiotoxicity. (Submitted)

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: SME-Specific Targeted Research Project (FP6)
EC contribution: € 2 933 291
Starting date: 1 January 2007
Duration: 36 months
Website: http://www.liintop.cnr.it

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 in 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 more 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. This will include genetic manipulation of existing cell lines (HepG2, HepaRG) which will be transfected with key transcription factors, in order to allow an appropriate expression of the differentiated phenotype. This will also include strategies to confer metabolic capabilities to other cell lines (e.g. Caco-2).

- Development of more complex cell co-culture models to combine absorption and metabolism in intestine 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 cell proliferation (e.g. cell cycle control, apoptosis/necrosis);

- effects on differentiated functions (e.g. protein secretion, cell junctions, expression of genes involved in transport and metabolism).

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

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

Experimental design

Concerning the availability of **functional hepatocytes** *in vitro* models, different approaches are in progress to obtain a functional *in vitro* model, and possibly some of them can be proposed at the end of the project as optimised or promising models. At the moment the HepaRG has been extensively characterised, showing relevant metabolic and transport properties of the liver. Moreover, it remains functionally stable for several weeks at confluence. Other approaches are related to genome modifications in order to preserve the relevant liver functions. One of these is dealing with the preparation of viral vectors for transfections of HepG2 with genes encoding for the transcription factors regulating the main metabolic enzymes (CYPs). The use of adenoviruses has shown to be effective, but with transient activity, while the use of lentiviruses may offer better possibilities, for the maintenance of the activity. The latter approach is under development. Another approach is the development of phenotypically stable and functional cultures of primary rat hepatocytes by using histone deacetylase (HDAC) inhibitors, e.g. Trichostatin A. Stable primary rat hepatocyte monolayer cultures have been achieved, which maintain a good cell morphology with prominent bile canaliculi even after seven days of culture, maintain CYP2B1 expression and activity to levels close to those observed in freshly isolated hepatocytes. Alternatively, postnatal progenitor cells could be used as source for the *in vitro* production of functional hepatocytes. Isolation protocols have been established to obtain rat and human mesenchymal progenitor cells (MPC) from the adult bone marrow, human skin progenitors cells (SKP) from adult dermis, and human adipose tissue stem cells (ADSC) from liposuction material and adult abdominal fat. The latter ADSC isolation protocol might need further optimisation to increase the isolation efficiency. Human MPC were able to differentiate into cells expressing a hepatic phenotype and functionality, upon sequential exposure to hepatogenic factors mimicking the secretion pattern during *in vivo* hepatogenesis and co-exposure to 1µM TSA (Table 1).

Table 1

Name of the test method	Hepatocytes for pharmacotoxicological investigations
Clinical endpoint	Hepatotoxicity and pathology.
Cell (line)	HepaRG, Transfected HepG2, TSA–treated primary rat hepatocytes and stem-cells derived hepatocytes.
Method description	The methods employed deal with induction and maintenance of the differentiated state in different models of hepatocyte in culture.
SOP	Yes (for stem cells isolation, TSA treatment, enzymatic profiling).
Endpoints	Steatogenesis, expression of CYPs, (by biochemical assays, western blotting, microscopy observations).
How is a positive result defined?	Profile of relevant markers, morphological characteristics.
How is a positive result expressed?	Performance of the models with respect to the *in vivo* situation.
Applicability	Compounds tested: steatogenic compounds and CYPs inducing compounds.
Positive control	Primary hepatocytes.
Negative control	Hepatoma HepG2 cells.
Performance	Sensitivity, specificity, accuracy, positive and negative predictive value.
Can the test method be used in a regulatory safety context?	No. This project deals with the development of optimised *in vitro* models and standardised procedure for their maintenance. Further testing should be performed on them to confirm the predictive value.
Which R would the test method impact?	Reduction/Replacement.
How can the test be used?	As part of an alternative/integrated testing strategy.

The **intestinal cells** are mainly represented by the human colon carcinoma cell line Caco-2. This cell line displays, in long term cultures, some characteristics of the small intestine: specialised functions such as specific brush border enzymes, absorptive and metabolic competence. However, there is no unique standard to compare with, due to differences in sources, maintenance and testing. Thus the different models have been compared at the level of the gene expression, protein synthesis, and functional activities. Principal component analysis demonstrated that culture conditions affect differentiation. Novel culture conditions have been experimented obtaining a higher degree of differentiation in comparison to the standard culture conditions. According to this

new method, the cell line express many of the intestinal epithelium differentiation markers at high level, as judged by microarray gene expression profile analysis and confirmed by quantitative real-time PCR. More work is in progress to better characterise the differentiation properties. Several enzymatic activities and transporters have already been detected and/or assayed (Table 2).

Table 2

Name of the test method	Enterocytes for pharmacotoxicological investigations
Clinical endpoint	Absorptive and biotransforming properties.
Cell (line)	Caco2 ATCC, Caco2 Inserm, Caco2 TC7.
Method description	The methods employed deal with induction and maintenance of the differentiated state in different models of CaCo2 cells in culture and the comparison of the line/clones used. TEER, brush border enzyme activity, Lucifer yellow test.
SOP	Yes.
Endpoints	Morphological characterisation, genome analysis, biochemical analysis of PhaseI and II biotransforming enzymes (e.g. gene expression. PCR, FACS, biochemical tests).
How is a positive result defined?	Profile of relevant markers, morphological characteristics.
How is a positive result expressed?	Performance of the models with respect to the *in vivo* situation.
Can the test method be used in a regulatory safety context?	No. This project deals with the development of optimised *in vitro* models and standardised procedure for their maintenance. Further testing should be performed on them to confirm the predictive value.
Which R would the test method impact?	Replacement/reduction.

6. Since the aim of the project is to obtain a well characterized cell line representative of the human small intestinal enterocytes, expressing the **absorptive and metabolic functions**, another set of studies has dealt with an initial characterisation of the transport activities expressed by Caco-2 cells, the parental cell lines, the clonal TC7 line and a patented model CacoReady. Permeability and transport experiment have been preliminary performed with in house compounds. The

characterisation so far completed has also confirmed that the Caco-2 cells express bio-transforming enzymes. Among them, UGT isoenzymes are currently under investigation as well as the CYP34 metabolism and Pgp efflux proteins. Moreover, an automated system to evaluate permeability has been set up and tested for passive permeation (high/low) and efflux activity. LC/MS methods were developed for a series of in-house compounds, glucuronides and sulphates, in order to be ready when the chosen common set of compounds will be tested by the partners in the second half of the project. In addition to that, a general method able to perform incubations, detect and quantify virtually any small molecule incubated in transport or metabolism experiment, has been developed and optimised, based on Ultra-high Pressure Liquid Chromatography and TOF spectrometer (Table 3).

Table 3

Name of the test method	Absorptive properties and metabolic activities in hepatocytes and enterocytes *in vitro*
Cell (line)	HepaRG, Caco2 ATCC, Caco2 Inserm, Caco2 TC7.
Method description	(HPLC/MS, biochemical assays).
SOP	Next step.
Endpoints	Molecules and metabolites determination.
How is a positive result defined?	With respect to the *in vivo* situation.
Applicability	Compounds tested: high, moderate and low passive permeability and active transport (ABCB1, SLC15A1).
Performance	Good *in vivo - in vitro* correlation.
Can the test method be used in a regulatory safety context?	Yes.
Which R would the test method impact?	Replacement/Reduction.
How can the test be used?	As part of an alternative/integrated testing strategy.

Concerning the **molecular and cellular targets** identification, a relatively simple mixture of three dyes (Hoechst 33342 for DNA content, TMRM for mitochondrial membrane potential and TOTO-3 for plasma membrane permeability) can be adopted in a high content screening based on confocal microscopy and HepG2 cells to detect toxic compounds (Table 4).

Table 4

Name of the test method	Toxicological tests
Clinical endpoint	Steatosis (for hepatocytes).
Cell (line)	Hepatocytes and Caco2.
Method description	Not yet available.
Endpoints	Cellular targets: membrane, mitochondria, cell death, cell replication.
Positive control	Primary hepatocytes.
Negative control	HepaG2 cells.
Which R would the test method impact?	Replacement/Reduction.
How can the test be used?	As part of an alternative/integrated testing strategy.

The ***in silico*** **modelling** was organised according to a four levels tiered approach: the first level constituting a base line to gather information on the performance of the various models. The further level is dealing with more specific approaches to be developed in parallel with the focusing on the specific cellular models. In the meantime, a preliminary *in vivo* database has been set up and a list of compounds relevant to metabolism or transport in the two types of cells, for which *in vivo* data are available, has been set up and agreed upon.

Results

Hepatic in vitro *models*

At least three hepatic *in vitro* models have been (or are) under development and characterisation:

- the human HepaRG cells, which are currently the most advanced in characterisation;
- the human HepG2 liver cell line, transfected with transcription factors/nuclear receptors/co-activators, regulating the main hepatic biotransformation activities, which is still under development;
- primary rat hepatocytes, exposed to HDAC inhibitors, to stabilise the hepatic differentiated phenotype, are fully developed; while the human postnatal progenitor cells-derived hepatocyte-like cells, are still under development.

Intestinal in vitro *models*

The partners involved in optimising and characterising Caco-2 cell line have been comparing, at the level of gene expression, the different lines used in the various laboratories. Genomic profiling data are now available for different culture conditions of Caco-2 INSERM and ATCC/LGC, for undifferentiated and differentiated cells, grown on filters or in flasks. The genomic profiles have already been compared to colonic epithelial cells, healthy and tumour. The

comparison with small intestine absorptive cells has been performed. At the same time, two genomic platforms are compared (Agilent and Affymetrix) in order to assess their performance.

Drug transport and metabolism capacity

Several partners have been involved in characterising some relevant bio-transforming Phase 1 and Phase 2 activities in those cells, and in setting up methodologies for measurement of drug metabolites. The main focus has been on CYPs and UGT enzymes, which have been characterised in the optimised cellular models of enterocytes and hepatocytes.

Limited to the role of human MDR1 (P-glycoprotein) an alternative cell line MDCK transfected with this transporter is evaluated as a tool to discriminate PGP substrates from non –PGP substrate. A P-gp (ABCB1) vesicular transport system and PEPT1 and OCT1 uptake transport assays have been developed.

Cellular and molecular targets as endpoints of drug exposure in intestine and liver

The idea of a multi-parametric assay integrating data from different indicators of cell function, obtained simultaneously, has been developed: it should be possible to classify compounds accordingly to their mechanism of action. Two analytical strategies could be envisaged: cytometric analysis by fluorescent probes, and metabonomics. A list of model hepatotoxins representative of the different molecular mechanisms of hepatotoxicity has been elaborated.

In silico *approaches for modelling hepatic and intestinal absorption and metabolism*

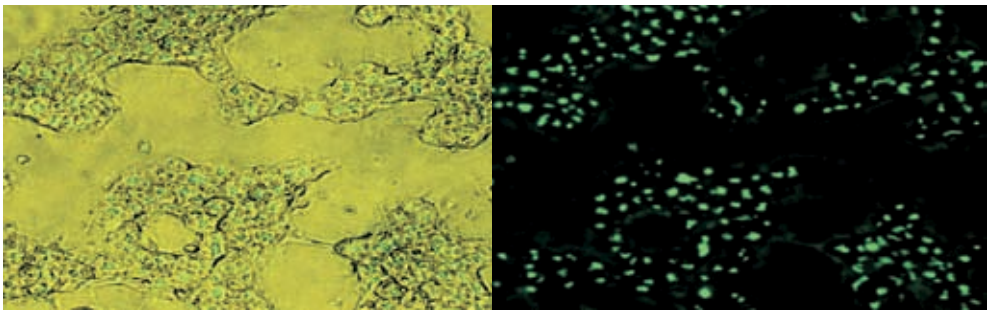
A list of compounds has been worked out by the partners, according to characteristics relevant to the cellular systems under study, and according to the knowledge available *in vivo*. A tiered approach of four levels has been proposed: the first 'in house' level has already been adopted in some laboratories and substances assayed for metabolism and absorption. This first level trial has also produced the setting up or the assessment of specific measurement methods.

Transfer potential of the developed in vitro *models for their utilisation within the industrial setting*

The SMEs are collaborating and transferring specific information and needs to the other partners within the cooperative framework.

Phase-contrast/Fluorescent microscopy

Fluorescence image



Functional activity of efflux transporters in differentiated HepaRG cells

Figure 1. Accumulation of carboxydichloro-fluorescein diacetate in bile canaliculi of HepaRG cells after 56 days of culture (From Guillouzo A and Guguen-Guillouzo C. 'Expert Opin', Drug Metab. Toxicol. 2008; 4, pp. 1279-94).

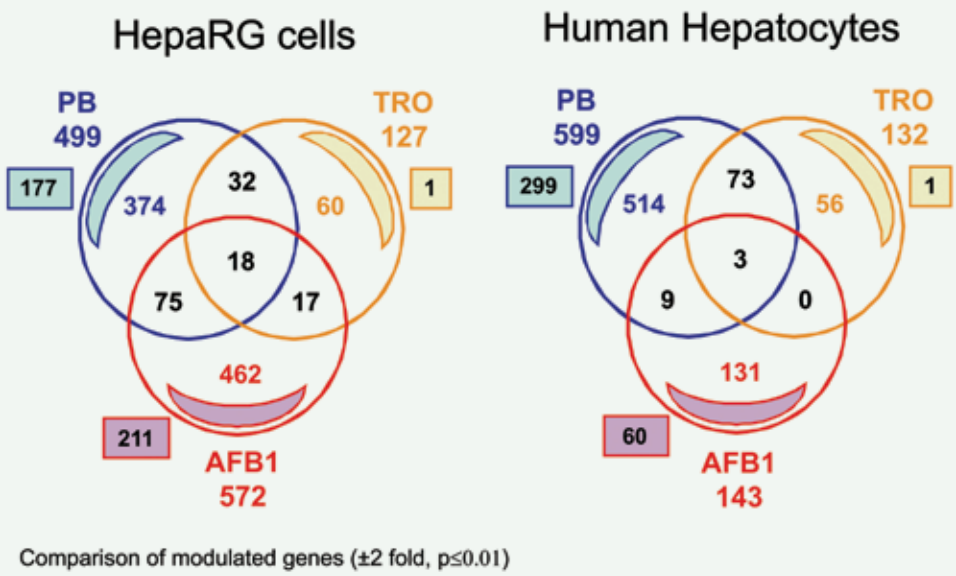


Figure 2. Venn diagram representation of differently expressed genes in HepaRG cells and primary human hepatocytes exposed to Phenobarbital (PB), troglitazone 4TRO) and aflatoxin B1 (AFB1) for 20h. One colour pangenomic Agilent microarrays. Anova analysis (Lambert et al., unpublished data).

Next steps

- The finalisation of the optimised cellular models of human hepatocytes and enterocytes reliable for prediction of drug absorption, metabolism and toxicity.
- The provision of sequential procedures, easily amenable to validation studies, possibly by miniaturised and automated technology.
- The provision of standards for these models, concerning *in vitro* absorption, metabolism and toxicity of selected drugs and the characterisation of relevant genes, especially involved in differentiation.
- The assessment of the performance of testing *in vivo* well known compounds on the *in vitro* models, with the help of mathematical modelling of pharmacokinetics and pharmacodynamics, developed on purpose.
- The development of a more complex cell co-culture model to combine absorption and metabolism in intestine and liver.
- Optimisation of the culture conditions to differentiate adult bone marrow stem cells into functional hepatocytes.

The updated **List of Publications** is available on the website (http://www.liintop.cnr.it), under 'Dissemination'.

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ARTEMIS

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

Contract number:	LSHB-CT-2006-037862
Project type:	SME - Specific Targeted Research Project (FP6)
EC contribution:	€ 1 984 900
Starting date:	1 March 2007
Duration:	36 months

Website: http://www.artemisproject.eu

Objectives

The objective of the project is the development of an *in vitro* system consisting of a bioartificial neural tissue made from mouse embryonic stem cells grown and differentiating to neurons inside hydrogel biomaterials that are interfaced with microelectrode arrays inside a bioreactor system, as seen in Figure 1. The synaptic network develops under electrical stimulation of the neurons transmitted through one of the electrodes. After the synaptic network has been developed, the response signal recorded from the other electrode will be stimulus-specific. This specificity corresponds to the 'memory' imprinted in the topology of the synaptic network.

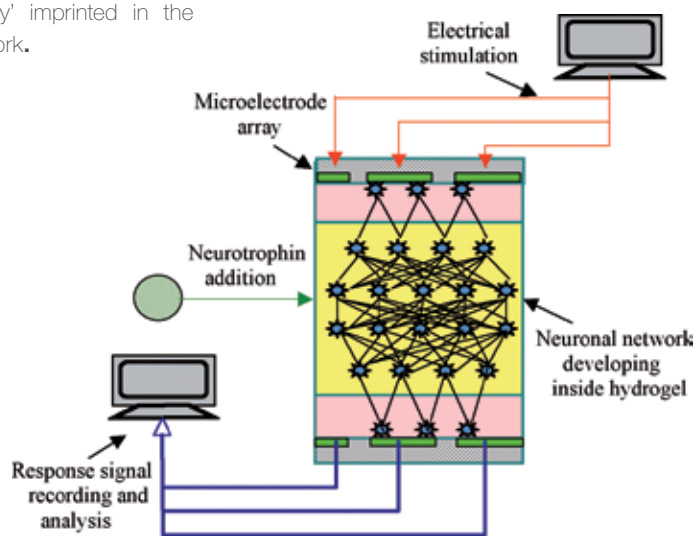

Figure 1. An *in vitro* neuronal tissue system that develops as *in vivo* with simultaneous electrical stimulation and neurotrophin addition for the development of a stimulus-specific synaptic network.

The system can be used to detect if neurotoxic compounds or drugs affect the memory. In addition it could be used as an *in vitro* disease model system to replace experiments in transgenic animals when the bio-artificial neural tissue develops from embryonic cells that have genes playing a role in memory switched-off.

Experimental design

Neurons are generated from embryonic stem cells using different differentiation protocols. Their properties are checked with the expression of gene markers and global gene expression analysis. Hydrogels in which the neurons can attach and grow are developed and their physical properties determined and optimised for the cell growth. Molecules that affect the cell attachment and dendrite development are used in the hydrogels in order to control the neurons' behaviour and synaptic network development. Perfusion bioreactor systems that carry the hydrogels seeded with neurons are installed and their operation is optimised to assure long term cell viability. Microelectrode arrays are incorporated in the bioreactors in contact with the neurons of the hydrogel. The electrodes are connected with a system for electrical stimulation, response signal amplification, noise extraction, spike detection and data storage, (MEA system). Algorithms for statistical analysis of the response signal are developed and installed in the system. The system has already been checked in experiments with neurons inside hydrogels. Signals have been recorded and analysed.

Results

We present below some of the results that are closely related with the evaluation of the system to be used for memory tests. The results are presented in the natural order used for the system, seen in Figure 1's assembly, i.e., from the generation of neurons, to their incorporation in hydrogels, to the incorporation of hydrogels in bioreactors and the recordings of the neurons electrical activity.

1. Neuronal differentiation in two dimensions

Mouse embryonic stem cells were cultured and differentiated into neurons according to two different protocols (Figure 2). In addition, mouse cortical neural stem cells have been used.

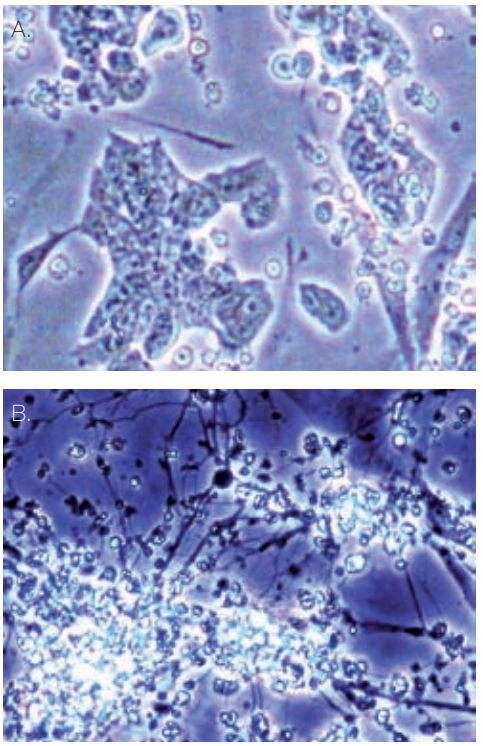

Figure 2. Mouse embryonic stem cells in expansion (a) and differentiation (b) phase growing in the form of colonies. Neurites started to be developed as seen in (b).

Fluorescence immunostaining was performed in various days of differentiation showing that the generated neurons express neuronal markers for a period of more than a month (Figure 3).

The presence of glial cells that secrete factors which modulate the synaptic transmission and enhance the formation of neuron connections, and therefore are important for the operation of the system, has been checked with immunostaining as seen in Figure 4.

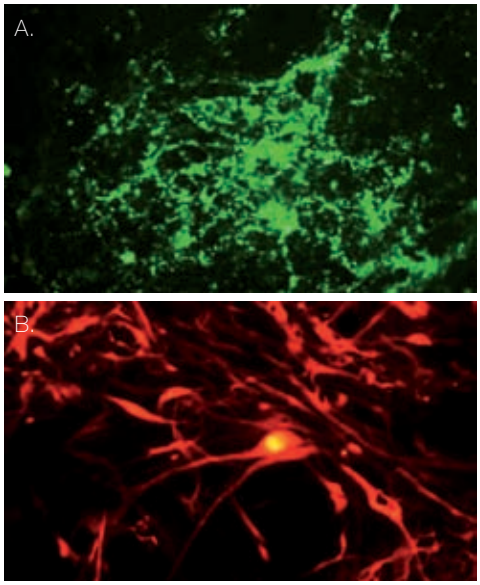

Figure 3. Synaptophysin (a) and nestin (b) immunostaining of neurons generated from mouse embryonic stem cells.

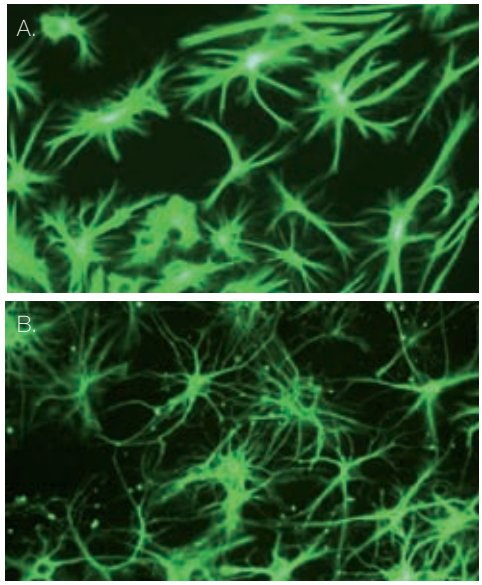

Figure 4. GFAP immunostaining of neurons generated from mouse embryonic stem cells for the detection of glial cells.

The functional maturation of the generated neurons was checked with gene expression analysis at different differentiation stages. The expression of critical receptors (NMDA, GABA), as well as the expression of genes involved in Long-Term-Potentiation (LTP), important for memory acquisition (LTP: neurons that fire together enhance their connectivity), has been confirmed.

2. Electrical activity of generated neurons

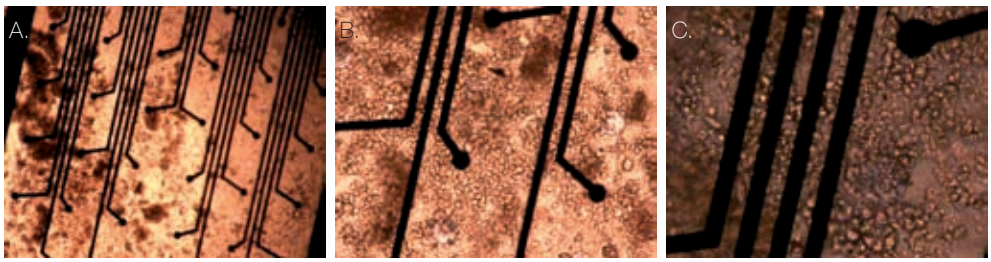

Figure 5. Neurons generated from mouse embryonic stem cells plated on a microelectrode array that has 32 microelectrodes, round black dots at the end of straight wires. The neurons attach and grow forming colonies (increasing magnification, a, 4X, b, 10X and c, 20X).

The electrical activity has been detected from neurons directly plated on the microelectrode array surface (Figure 5), as well as neurons inside hydrogels interfaced with electrodes. Recordings have been taken from the microelectrode array as shown in Figure 6.

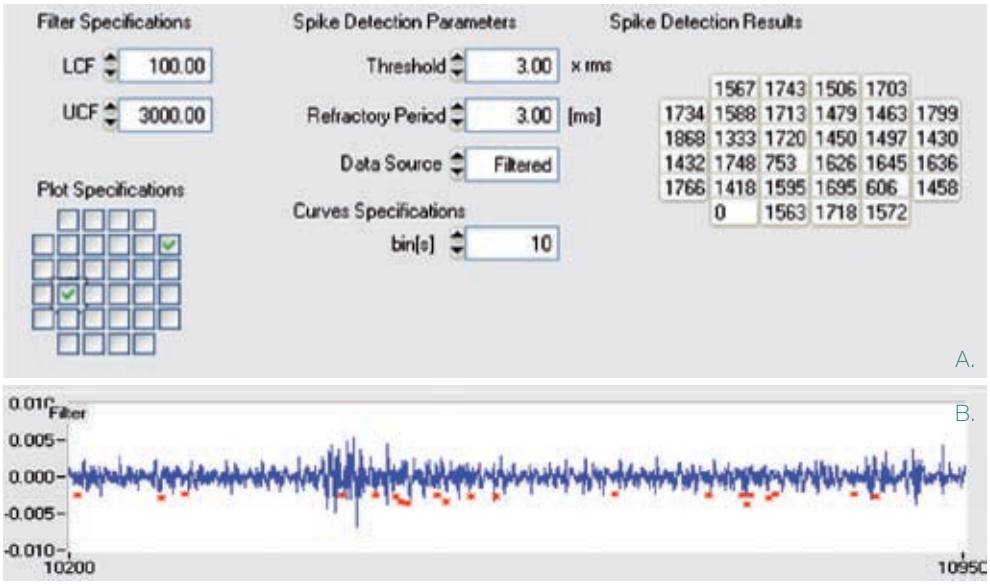

Figure 6. Screening of spikes, numbers in the boxes, detected in one minute by each one of the 32 microelectrodes of the array (a), shows high activity. Part of the recorded signal from one of the microelectrodes (b). Spikes are detected after removing the noise of the signals and are indicated with red dots in (b).

3. Neurons inside hydrogels

A high number of hydrogels has been tested for cell attachment. The best hydrogels found were the ones with positively charged surface. Porosity has been optimised to sustain the viability of a high number of cells, needed for the development of a continuous synaptic network, as seen in Figure 7.

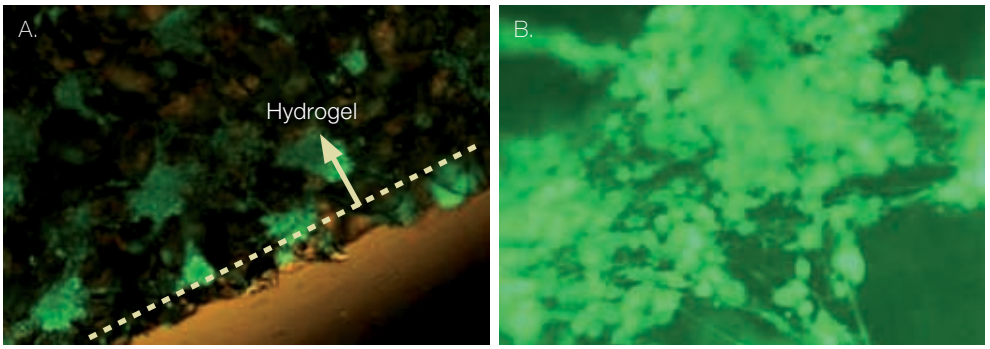

Figure 7. Mouse cortical neural stem cells grown forming interconnected patches on porous hydrogel, (a). Cell patches have a high number of synaptically interconnected cells, (b). (Calcein fluorescence staining of alive cells, a, 4X and b, 20X magnification).

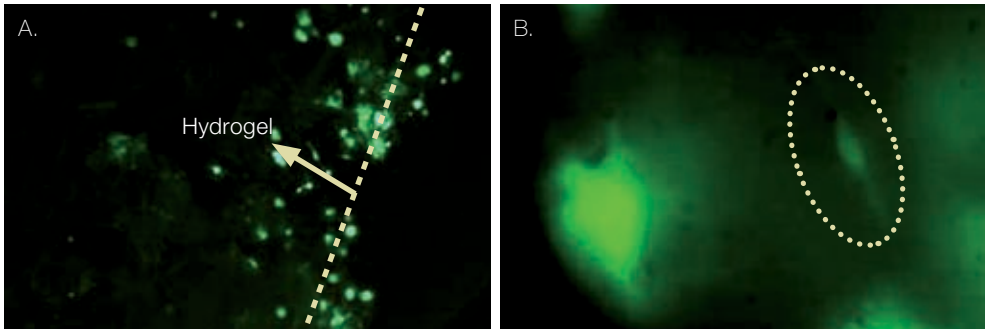

Figure 8. Neurons generated from mouse embryonic stem cells distributed in the form of small aggregates (a). A cell with neurite extension can be seen in (b). (Calcein fluorescence staining of alive cells 4X (a) and 20X (b) magnification).

4. Neurons inside functionalised hydrogels

To further enhance the development of the synaptic network, the hydrogels have been functionalised with molecules that enhance neurite outgrowth (Figure 9a), or with molecules that facilitate cell attachment as well as neurite development (Figure 9b).

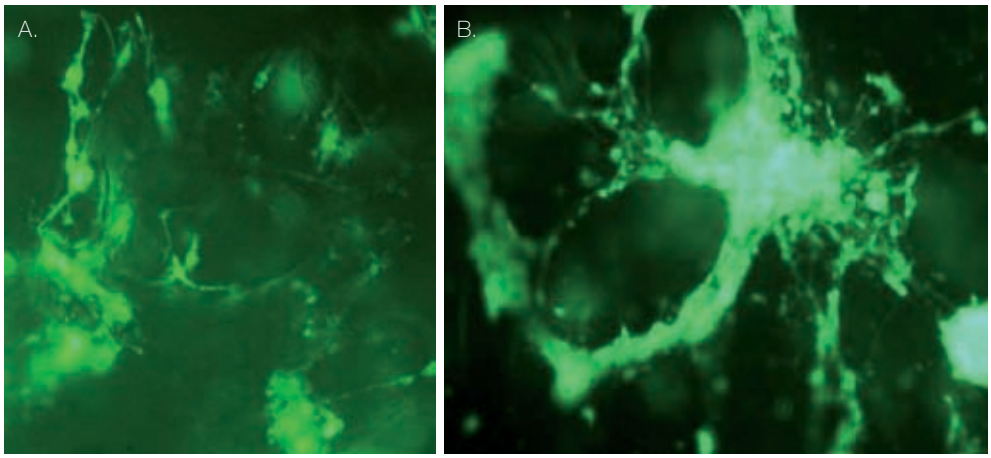

Figure 9. Mouse cortical neural stem cells grown forming extensive synaptic connections on porous functionalised hydrogels. (Calcein fluorescence staining of alive cells 20X (a) and 40X (b) magnification).

5. Hydrogels/neurons interfaced with electrodes in perfusion bioreactors

A perfusion bioreactor was used for long term cultures of neurons contained in the hydrogels, (Figure 10). Two electrodes, stimulation and a recording one, have been interfaced with the hydrogel. Oxygenation is allowed through permeable tubes that perfuse the medium. In order to stabilise the relative position of the hydrogel and electrodes in order to assure a good and permanent contact of the microelectrodes with the neurons, two discs of a porous hydrogel act as support exerting pressure on the hydrogel/ electrodes construction.

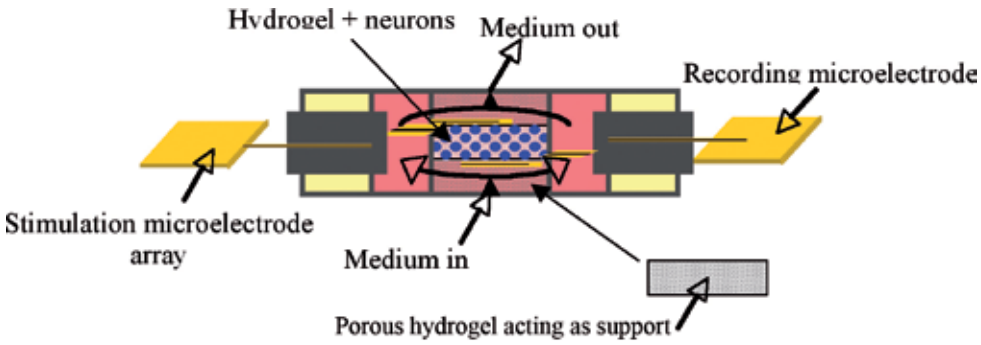

Figure 10. Cross section of the perfusion bioreactor with hydrogels containing neurons interfaced with two microelectrode arrays.

Neurons embedded in hydrogels exhibited similar electrical activity as neurons directly plated on the microelectrode array surface (Figure 11). The trend in electrical activity along the differentiation days is to rise reaching a peak close to day 23. After reaching the peak, the electrical activity drops. The initial rise of the electrical activity is due to the increase in the cell number (as differentiation continuous, the cells also grow though the growth gradually decreases), since each microelectrode receives signals from several cells attached on it or in the surrounding area spreading neurites that reach it. The most probable reason for the drop of the electrical activity in later differentiation days is the overgrowth of cell colonies resulting in limited availability of nutrients and oxygen to the internal cells, which then start dying. We have therefore determined the useful time for which the system could be reliably used for tests.

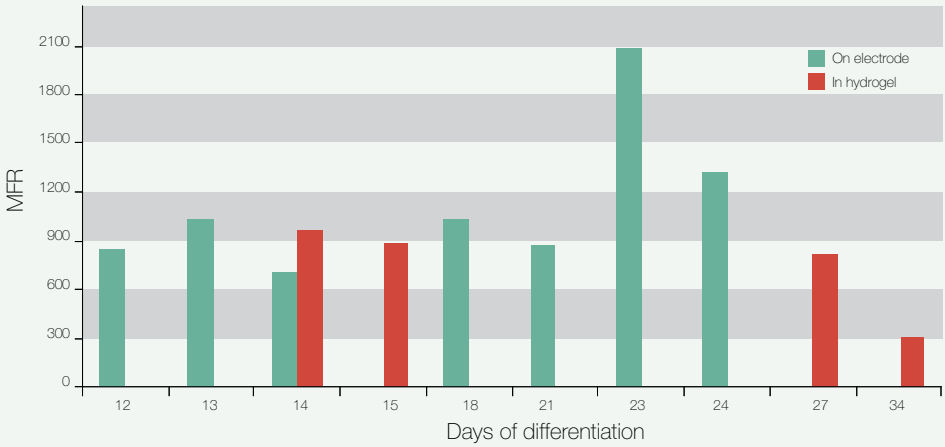

Figure 11. Average electrical activity of the 32 microelectrodes of the array in different differentiation days from neurons either plated on the array or inside hydrogel interfaced with electrodes. (MFR, Mean Firing Rate, MFR, the number of spikes detected by all the microelectrodes divided by the number of microelectrodes).

Next steps

In the second phase, neurons generated from normal as well as from transgenic embryonic stem cell lines that have been already developed will be used in the system to compare the normal and transgenic *in vitro* developing neural tissue. The ability of the defective (transgenic) synaptic networks to memorise the electrical stimulus will be tested from the analysis of the signal with which the neural tissue responds to a 'memorised' stimulus. 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, selected during the first period of the project, will be performed in the system and their effect on the ability of a normal synaptic network to memorise electrical stimuli under their influence 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 that however could not be extrapolated to behavioural effects referring to high level functions such as memory.

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

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ESNATS

Embryonic stem cell-based novel alternative testing strategies

Grant agreement number: HEALTH-F5-2008-201619
Project type: Collaborative Project (Large-scale integrating project) (FP7)
EC contribution: € 11 895 577
Starting date: 1 April 2008
Duration: 60 months
Website: http://www.esnats.eu

Objectives

The aim of the ESNATS project is to develop a novel toxicity test platform based on embryonic stem cells (ESCs), in particular human ESC (hESCs), to streamline the drug development R&D process and evaluation of drug toxicity in clinical studies, reduce related costs and thus not only increase the safety of patients but also reduce the number of animals due to earlier detection of adverse effects.

ESNATS addresses current shortcomings in toxicity testing:

- A major part of safety testing takes place late in the research and development cycle, implying protracted experimentation involving high numbers of animals and generating significant costs.
- Some *in vitro* assays rely on cells 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 potential of ESCs, including:

- their capacity to self-renew, constituting a potentially unlimited source of cells;
- their pluripotency, providing a source for cells of different phenotypes required for toxicity testing;

- the physiological relevance of ESC-derived somatic cells for toxicity endpoints, offering a perspective of tests with improved predictivity;

- at least for murine ESCs (mESCs), their easy genetic manipulation, allowing use of reporter gene expression as a powerful toxicity testing tool.

To reach the project goals, a battery of toxicity tests is being developed using ESC lines subjected to standardised culture and differentiation protocols. Tests will cover ESCs in several stages of development as well as differentiated derivatives, including gamete and neuronal lineages, complemented with systems for hepatic metabolism. Genomics approaches will be used to determine predictive toxicoproteomics and toxicogenomics signatures. The individual tests will be integrated into an 'all-in-one' testing strategy. To ensure practical usage in the pharmaceutical industry, concepts for automated ESC culture will be developed and the test systems will be scaled up. In a later stage of the project, the predictivity, quality and reproducibility of the test strategy will be evaluated in a 'proof of concept' study.

The results of ESNATS are expected to have an impact at several levels:

- on pharmaceutical R&D, by providing a new technology which will facilitate screening and early decision-making of candidate drugs, and in the long-term might contribute to a more rationale 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 stable hESC culture, improved protocols for hESC differentiation and a world leading toxicogenomic database.

Furthermore, the new testing rules under the European Regulation of Chemical Substances (REACH) require extensive toxicological safety testing of both existing and new chemicals 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 framework.

Note about the use of human Embryonic Stem Cells in ESNATS

The ESNATS scientific programme touches on several ethical issues, in particular concerning animal research and human embryonic stem cells. These were recognised from the outset. For this reason a specialist ethical organisation, Edinethics Ltd, was appointed as a partner in the consortium and to the Steering Committee to provide ethical advice and training.

ESNATS seeks to address one notable area of animal experimentation, that of replacing the use of animals by hESCs for a range of testing procedures for the toxic effects of pharmaceuticals and other chemicals especially on human reproduction and development (reproductive toxicology) and on the human nervous system (neuro-toxicology). By using human cells, the programme aims to achieve two ethical advantages:

- one is to do the basic research on stem cell propagation and differentiation, and applied research on validation and quality control, which will enable tests to be developed which should support the reduction of animal experimentation in testing strategies;

- the second is to lead to a better quality type of toxicological data, by relying on the response of human cells rather than animals.

Ethically set against the use of embryonic stem cells, however, is the need to use human embryos to derive the human cells. This is a matter of sincere but deep disagreement across Europe, among and within the EU Member States, and their legislation reflects it with varying levels of what is or is not permitted.

The EC FP7 research programme does not fund research involving the *de novo* derivation of embryonic stem cells; only projects that use existing hES cell lines that have been derived from donated embryos which were originally created with the aim of conceiving children by medically assisted procreation, but which the couple do not now intend to implant, can be funded. These so-called 'surplus' embryos are a maximum of five to seven days old. The couple gives their written informed consent to donate for stem cell research. If the embryos were not donated they would eventually have been destroyed. Ethically this is generally seen as the most acceptable circumstances under which embryos might be used, a kind of 'lesser of two evils', for those who would object to creating embryos specifically for research.

With current methods, stem cells are isolated from the inner cell mass of the embryo and are subsequently cultured in advanced cell cultures. Once isolated, these cells cannot by themselves give rise to a pregnancy.

The ESNATS project will not use cells derived from human embryos which were created explicitly for research from human gametes, or from any human embryos created by cell nuclear transfer cloning methods of any kind, or from parthenogenetically derived human embryonic material. Furthermore, ESNATS does not establish new human embryonic stem cell lines since the consortium has agreed on the use of existing cell lines.

Experimental design

The ESNATS project is developing test systems based on hESCs. For most tests, ESCs will not be used directly but have to undergo differentiation in a standardised way in order to test effects of a substance either on development or on ESC-derived, tissue-specific cells. During the current first stage of the project, appropriate, standardised differentiation protocols are being developed. At this stage of the project, the framework for data gathering and analysis is set up, and reference compounds and industry and regulatory requirements are identified.

Once protocols for ESC differentiation are established, development of the following test systems is planned:

- *in vitro* gametogenesis from murine ESCs for toxicological testing;
- preimplantation embryotoxicity tests based on undifferentiated ESCs;
- preimplantation embryotoxicity tests based on hESC-derived trophoblast models;
- humanised teratogenicity *in vitro* test for drug safety screening using neural and cardiac differentiation assays based on hESCs;
- hESC-based predictive epigenetic profiling and pluripotency reporter screening systems;
- hESC-based early human developmental toxicity test for increased throughput testing in early drug discovery;
- developmental neurotoxicity;
- acute and subacute neurotoxicity;

- functional toxicity in integrated neural tissue;
- developmental toxicogenomics signatures.

Development of these test systems will last for the first three project years. During the last two years, the consortium will then bring together the different assays developed in a test battery, and focus on the pre-validation of the testing strategies.

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.

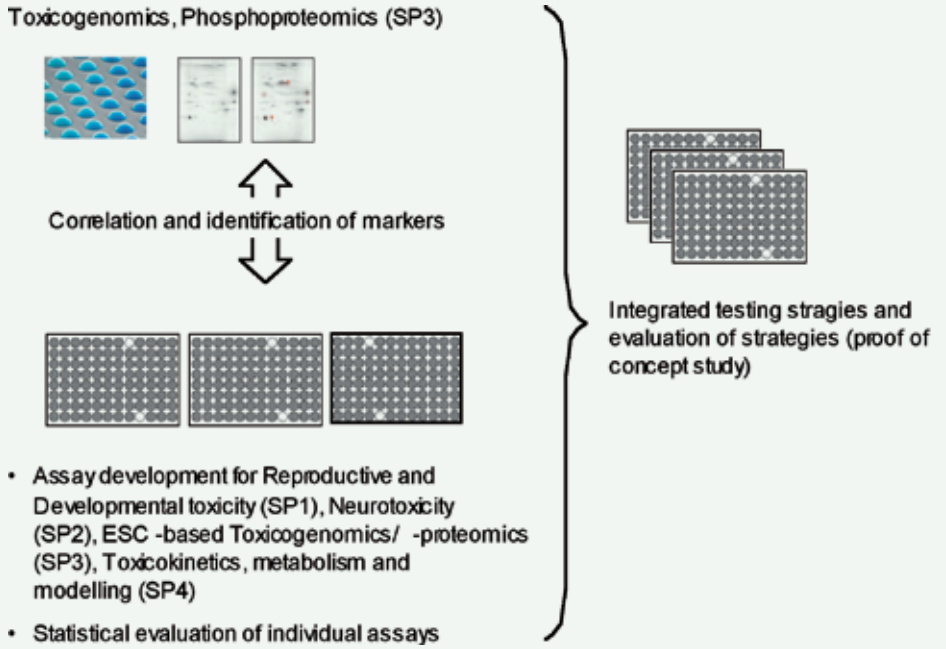

Figure 1. ESNATS' main research areas.

SP1 Reproductive Toxicity

Sub-project 1 is focussed on the development of ESC-based *in vitro* models for evaluating the hazard of compounds to the mammalian reproductive cycle. SP1 will provide more detailed information on the toxicological mechanisms in different sensitive phases of the reproductive cycle, such as germ cell development, preimplantation development and early embryonic development.

SP2 Neurotoxicity

Sub-project 2 aims to develop reliable and cost-efficient test systems to evaluate the effect of compounds on neuronal development and neuronal viability and functionality. An integrated approach is proposed to develop an ESC-based system for neurotoxicity testing, which will allow overcoming obstacles such as variability in the cell lines and culture conditions, the influence of the mode of neuronal ESC differentiation, poor or irrelevant read-outs and extrapolation of *in vitro* toxicity to *in vivo* toxicity.

SP3 ESC-based toxicogenomics and toxicoproteomics signatures

The overall goal of SP3 is to investigate the influence of compounds on gene expression and proteomics. SP3 will therefore develop *in vitro* toxicity models based on high throughput genomics methods by identifying specific toxicogenomic and phosphoproteomic signatures of drug candidates on the developmental processes of ESC towards tissue specific cells and on the ESC-derived neurons, spermatocytes and hepatocytes.

SP4 Metabolism, toxicokinetics and modelling

A major difficulty in evaluating a toxicological risk *in vitro* is accounting for an *in vivo* like metabolism. This SP therefore deals with the integration of metabolising systems into the ESNATS testing approaches for neural and reproductive toxicity.

Besides comprehensive knowledge about metabolism, SP4 will also contribute with physiologically based pharmacokinetic information, i.e. the concentration of test substances and their metabolites at the target cells within the organism.

cWPs cover the following transversal topics:

- the requirements of stakeholders (pharmaceutical industry, regulators and ethicians) in terms of innovative evidence based toxicity testing will be gathered in cWP1 'Steering committee';
- cWP2 'Knowledge Management, Validation and Testing Strategies' ensures systematic management of knowledge and evaluation of results;
- the bottleneck of provision of standardised cells (hESCs and hESC-derived cells) will be addressed in cWP3 ('Methods for automating scale up of stem cell production and cell banking').

Results

At this stage of the ESNATS project, test systems are still under development. The following achievements have been reached so far:

- optimised protocols for murine ESC-derived *in vitro* gametogenesis;
- creation of undifferentiated reporter hESC cell lines;
- Standard Operating Procedure (SOP) for hESC neural differentiation;
- reliable techniques for the transfer of defined populations of hESC to 96 well plate format;
- methods for differentiation of neural precursor cells in monolayer cultures;
- SOP for murine neural precursor cells (mNPCs);
- first SOP for engineered neural tissues;
- publication of commented list of neurotoxicity reference compounds;
- compilation of a protocol and tool compound list for prevalidation of mature ESC-derived neurons as *in vitro* alternative method for toxicity testing;
- selection of optimal endpoints/endpoint combinations to detect neurotoxicity with high predictive capacity for the *in vivo* situation;
- report on possible readouts of synaptic density in engineered neural tissues.

Next steps

- Development of individual tests;
- Assessment of robustness and reliability of tests in intra-laboratory studies;
- Comparative analysis of individual toxicogenomics data to identify specific toxicogenomics signatures;
- Integration of individual tests into a testing strategy.

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2.2 Integrated testing strategies

The project presented here, ACuteTox, aims to develop and prevalidate a testing strategy for the prediction of human acute systemic toxicity. It integrates *in vivo* and *in vitro* data from individual assays and test batteries. It takes into account factors such as absorption, distribution, metabolism and excretion (ADME) and organ specificity.

ACuteTox

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

Contract number: LSHB-CT-2004-512051
Project type: Integrated Project (FP6)
EC contribution: € 9 000 000
Starting date: 1 January 2005
Duration: 60 months
Website: http://www.acutetox.org

Objectives

The ACuteTox project aims to develop and prevalidate a simple and robust *in vitro* testing strategy for the prediction of human acute systemic toxicity. This has the potential to replace the animal acute toxicity tests that are currently used for regulatory purposes. 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 LD50 values. Moreover, the MEIC (Multicentre Evaluation of *In vitro* Cytotoxicity) programme showed a good correlation (around 70%) between *in vitro* basal cytotoxicity data and human lethal blood concentrations. 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 systemic toxicity. The project is divided into nine scientific work packages:

1. The generation of a high quality *in vivo* database;
2. The generation of a high quality *in vitro* database;
3. Iterative amendment of the testing strategy;
4. New cell systems and new endpoints;
5. Alerts and correctors in toxicity screening (I): Role of ADE;
6. Alerts and correctors in toxicity screening (II): Role of metabolism;

7. Alerts and correctors in toxicity screening (III): Role of target organ toxicity such as neuro, nephro and hepatotoxicity;

8. Technical optimisation of the amended test strategy;

9. Pre-validation of the test strategy.

The overall scientific objectives of the project are:

1. Compilation, critical evaluation and generation of high quality *in vitro* and *in vivo* data for comparative analysis;

2. Identification of factors (absorption, distribution, excretion, metabolism and organ specificity) that influence the correlation between *in vitro* toxicity (concentration) and *in vivo* toxicity (dosage), and the definition of an algorithm that accounts for this;

3. Exploration of innovative tools and cellular systems to identify new end-points and strategies to better anticipate animal and human toxicity;

4. Design of a simple, robust and reliable *in vitro* test strategy amenable for robotic testing, associated with the prediction model for acute toxicity.

Experimental design

Known outliers of available *in vitro* - *in vivo* correlations are being evaluated in order to introduce further parameters that might improve the correlation, including absorption, distribution and excretion, metabolism and organ specificity. Reference chemicals (1) selected mainly from previous studies (MEIC and ECVAM/ICCVAM validation, for example) are being tested in different *in vitro* and *in silico* assays (2). This allows for the integration of alerts in a prediction algorithm, which together with a robust implementation of medium-throughput approaches, would enable the creation of a new testing strategy with better prediction for toxicity classification. The project is being carried out over a five-year period; the testing strategy will be prevalidated in the last year.

Expected outcome

The full validation of the final testing strategy will lead to regulatory approval and its incorporation into the set of standardised test guidelines for chemical hazard assessment. The proposed testing strategy could potentially replace EU methods B.1bis and B.1tris in Annex V of Dir 67/548 EEC and, subsequently, the corresponding OECD Test Guidelines 420, 423 and 425.

Results

Animal and human data for the 97 ACuteTox reference compounds were compiled (1, 25). The database contains LD50 values from animal studies, as well as human data from case reports, including acute sub-lethal and lethal blood (LC) concentration data. Descriptive summaries containing physico-chemical data, LD50 (lethal dose 50%) values, human toxicity data, pharmacokinetics-/toxicokinetics- data, metabolism, toxicological mechanisms, and target organs for all 97 reference chemicals have been recorded.

A statistical evaluation of the animal data collected indicated good reproducibility of LD50 values for the vast majority chemicals. Furthermore, rat and mouse mean LD50 were highly correlated with two exceptions: warfarin and cycloheximide were much more toxic in rat. Regression analysis of human acute lethal concentrations with rat oral LD50 data for 30 reference chemicals resulted in a coherent correlation with slope 0.955, intercept -0.615, and coefficient of determination 0.571, which was similar to the results obtained in the MEIC study (26).

The testing of the ACuteTox reference chemicals in six basal cytotoxicity tests has been completed. Data for most of the 97 reference compounds, that passed the strict assay acceptance criteria, are available for the Fa32/NR uptake, Fa32/protein content, 3T3/NR uptake and NHK/NR uptake assays. Data are available for half of the compounds in the HepG2/protein content and HL60/ATP content systems. All six basal cytotoxicity assays give similar results, which confirm the results from the MEIC study. The toxicity ranking of the compounds is the same when the cell types are compared, with the exception of colchicine, cyclohexamide, hexachlorobenzene, digoxin and 5-fluorouracil (1). The Hep-G2, HL-60 and NHK cells showed a somewhat lower sensitivity i.e. higher IC50 (inhibitory concentration 50%) data than the 3T3 and Fa32 cells.

All *in vivo* data collected as well as all *in vitro* data obtained in the project are stored in Acutoxbase, a database which was developed to facilitate Standard Operating Procedures (SOPs) storage, data transfer from all partners and statistical analysis of larger data sets. At the end of 2008 Acutoxbase contains a full set of data regarding the selected 97 reference chemicals, including molecular structure, physicochemical properties and summary descriptions on use, toxicity and *in vivo* biokinetics. Moreover, about 2206 data from acute oral toxicity studies *in vivo*, 2902 data sets from human blood poisoning reports, 10300 files from *in vitro* experiments and 90 different SOPs for *in vitro* assays were introduced into the database. The *in vitro* and *in vivo* data (both the summary, as well as the raw data) can be easily retrieved as Excel files and used for any type of analysis (e.g. statistical calculations, comparisons etc.) (2).

One of the aims of the project is to adapt the methods of the testing strategy to high throughput screening (HTS). The 3T3/NR uptake and HepG2/MTT assays have successfully been adapted to two commercially available HTS robotic platforms.

In vitro data (IC50-values) of the 97 ACuteTox reference chemical obtained in the 6 basal cytotoxicity assays and the collected *in vivo* data (LD50 and LC50 values) were compared using multivariate analyses with the aim of identifying additional outliers that have been tested by the Partners before the selection of methods that will enter the prevalidation phase. The models based on the 3T3 assay and LD50 or human LC50 values were used to identify outliers by use of normal probability plots. With the LD50 model 14 outliers were identified and with the human LC50 model, 16 were identified. However, the model for the LC50 values in humans were better compared to LD50 values in rat.

Fifty-seven reference chemicals (3) have been tested in selected assays including 77 end-points (Table 1) and the results have been reported in Acutoxbase. These data sets have been integrated in the *in vitro - in vivo* comparison with the aim of selecting methods for improving the correlation and thus becoming candidates for inclusion in the testing strategy (see below).

In addition, in order to investigate an alternative way for improving the prediction of acute toxicity, the project evaluated the use of more specific end-point parameters, and/or cell models from the haematopoietic system in the testing strategy. Effects on the *in vitro* production of cytokines in whole human blood cultures (4) as well as effects on the CFU-GM and progenitors of megakaryocytes (3) have been measured for 56 chemicals and the results showed a very good correlation with the rat oral LD50 values (R2 around 0.85). A novel assay of cytokine secretion have been developed by using human whole blood, based on the detection by bead-based flow cytometry of three relevant inflammatory cytokines; IL-1β, IL-6 and TNF-α upon stimulation of leucocytes by a bacterial product, lipopolysaccharide. In this way, the effect of toxic exposure on immune function may be examined through a more relevant method than the *in vitro* models based upon activation induced by a mitogenic agent, such as phytohemagglutinin.

By performing miniaturised assays (grouped as Cytomic Panel for Cytotoxicity Screening and Cytomic Panel for Oxidative Stress Screening) with 56 chemicals in 3 human cell lines (HepG2 hepatoma, SH-SY5Y neuroblastoma and A.704 kidney adenocarcinoma) it was shown that the cytomic assays correlated excellently with *in vivo* human toxicity, lower with *in vitro* and very poor with rodent toxicities (5-8). The suitability of these assays for classification, according to the Global Harmonization System (GHS) was assessed. The result showed that the cytomic assays do not separate clearly compounds belonging to toxic classes (GHS classes 1-5) but seem to reveal compounds labelled as non-toxic by GHS. It was concluded that cytomics is a promising analytical system for ACuteTox and for similar *in vitro* cell-based toxicological studies.

The most crucial parts of the kinetic behaviour have been studied. For this purpose, the determination of kinetic parameters is being performed either by experimental, *in vitro* tests or computer-based kinetic modelling. Neural network models for oral absorption and BBB passage classify the compounds with 73% and 72% accuracy as compared to the *in vitro* models, respectively (9). Results from the Caco-2 models for prediction of oral absorption, used in three different laboratories, have been compared. The results show good agreement between the different variants. Toxicity studies and permeability studies using *in vitro* BBB models have been performed for the comparison of permeability coefficients (Papp-values) in different Caco-2 systems showed that although the absolute values were different, the rank order of the Papp values was strikingly comparable. Furthermore, based on the original BBB *in vitro* model, a new BBB *in vitro* model has been developed to fit with the needs when screening large numbers of compounds (10).

Another aim was to investigate the partitioning behaviour of a number of polycyclic aromatic hydrocarbons (PAHs) to different components of a typical *in vitro* assay by using solid phase micro-extraction. This technique was proven to accurately measure the free concentration of compounds such as PAHs, while being easy to use. Finally, *in vitro* plasma protein binding of reference compounds was performed.

A set of rules or alerts has been developed to identify those chemicals for which one or more of these processes may lead to a reduction of the actual or bioavailable concentration in the *in vitro* cytotoxicity assay. These alerts are based on physical and chemical properties, including protein binding affinity and the octanol-water and air-water partition coefficients. Based on these rules, problematic chemicals from the list of 97 chemicals have been identified (11). The data obtained from these studies are the basis for the biokinetic modelling. Preliminary results indicate that integration of kinetic information, such as absorption, protein binding, lipophilicity and clearance with cytotoxicity test results could improve the prediction of acute systemic toxicity.

In order to evaluate if toxicity is dependent on metabolism, the effects of the 57 reference compounds as well as five additional bioactivable compounds have been compared between a metabolic competent model (primary hepatocytes) and a non-metabolising cell type (HepG2) by use of MTT. By comparing the concentration-toxicity curves of each compound in both models it is possible to ascertain whether the molecule elicits toxic effects preferentially on hepatocytes suggesting that a bioactivation of the xenobiotic is required. To examine the robustness of this strategy, intra-assay, inter-assay as well as intralaboratory variability was investigated for each cell system. A low variability (%CV<10%), both intra-plate and intra-assay, was obtained in all laboratories, however, the intra laboratory variability needs to be improved. The higher intra-laboratory variation could be due to several reasons, for example the physicochemical properties of the compounds assayed, many showing hydrophobicity and poor water solubility.

The Adeno-CYP HepG2 model developed earlier within the project (12-13) has been adapted to 96-well plates. Tamoxifen, tetracycline, cyclosporine A, amiodarone, atropine sulphate, verapamil-HCl and SLS have been tested in the new ready-to-use models (adenoCYP3A4- and 2E1-HepG2). Data on protein binding and metabolic stability (using rat and human liver microsomes and/or human and rat hepatocytes) have been generated for a sub-set of the reference compounds.

Another aim was to investigate how METEOR and DEREK software performs in prediction of metabolic fate of compounds with known biotransformation. Fourteen ACuteTox reference compounds were tested, indicating that METEOR appears to be an interesting alternative for *in silico* prediction of metabolism. Furthermore, it was concluded that even if the DEREK programme does not predict acute toxicity *per se*, important information regarding the toxic profile of the tested substances can be gained.

In the neurotoxicity experiments native or differentiated human neuroblastoma SH-SY5Y cells, primary cultures of mouse or rat neurons, and mature re-aggregated rat brain cells were used (14-20). The results show that the broad collection of assays could, in a very good way, predict the neurotoxic compounds. However, the evaluation of data showed that no single neuronal endpoint (AChE activity measurements, GABAA receptor function, CMP, CASP3, RS, GUp, NF-H, GFAP and MBP) dramatically improved correlation to the human lethal blood concentration (used as an estimate of the target tissue concentration of acute systemic toxicity) as compared to the basal cytotoxicity measured in the 3T3-NRU assay when analysed by linear regression. However, the neurotoxic endpoints identified several 'alerts', defined as a displayed effect in the neuronal endpoints at lower concentrations than in the 3T3-NRU test. Hence, the combination of basal cytotoxicity data (IC50) with neurotoxicity data (NTC) gave a better prediction of the LC50 than IC50 alone. All neuronal endpoints identified a few chemicals with higher predicted activity (over-estimated toxicity) than the LC50. This can, in some cases, be explained by a restricted passage over the blood brain barrier, which illustrates the importance of integrating biokinetic information in the prediction of systemic toxicity by the use of the *in vitro* methodology.

For the measurement of nephrotoxicity, transepithelial resistance (TEER) was chosen as the functional assay and the LLC-PK1 proximal tubular cell line as the test system. The functional assay reflects the *in vivo* transporting capabilities of the renal proximal tubules. The functional assay was compared to a viability assay namely the resazurin (alamar blue) assay under exactly the same experimental conditions and testing was carried out in the 96-well plate format for both assays.

The REMS automated device was selected for measurement of TEER. The 57 reference chemicals (including some nephrotoxic) were tested and the overall results show that the TEER is a sensitive predictor of toxicity. Using the data obtained, the IC20, IC50 and IC80 for both assays for all chemicals tested were calculated, where possible, using the statistical analysis techniques available at present. TEER showed greater sensitivity for nephrotoxic chemicals compared to non-nephrotoxic chemicals. However, compounds requiring metabolism, such as diethylene glycol did not show toxicity at the highest concentration tested. Excellent inter-laboratory comparison of the TEER was obtained between the two participating laboratories. The results indicate that the TEER functional assay is a very promising assay to detect nephrotoxicity *in vitro* and is more sensitive than a viability assay. The REMS automated device facilitates high throughput of the TEER assay.

The main goal of hepatoxicity experiments has been to identify a set of markers characteristic of acute liver toxicity that could be of use in high throughput screening. Metabolic competent cells (rat hepatocytes), non-competent hepatic cells

(HepG2) and non hepatic cells (3T3 fibroblasts) were exposed to the 57 reference compounds using the MTT assay (21, 22). The data will be analysed as soon as the new software Acusoft is ready. Acusoft is a further development of Phototox software with the aim to fit the needs of the ACuteTox project, such as comparing concentration-response curves from three different cell cultures. A first version of the software was developed during 2008. Experimental data can automatically be imported to the software from any type of Excel sheets. For each compound tested in the study, the software calculates final results such as % viability and ICs automatically. In addition, the software allows for the calculation of the relative IC50 of the test compound considering the IC50 values of low and high reference compounds. This will enable better comparisons of IC50 between compounds.

By loading the plasma membranes with cholesterol, it was possible to increase the reproducibility of the ATP hydrolysis of Bsep assay (23). However, the signal-to-noise ratio is not satisfactory and the conclusion is that this ATPase assay will not be considered for the prevalidation phase of ACuteTox. Two fluorescent bile acid derivatives (namely, 3a and 7a NBD substituted) have been used with a subset of cholestasic and non-cholestasic compounds. The work has provided a proof of concept of this assay and the results are now being analysed (24).

Overall conclusion and next steps

The *in vitro – in vivo* modelling of LC50 blood concentration values for humans and LD50 values (25) has been a central activity of the project during 2008 (3). Six of these variables included in the analysis are basal cytotoxicity tests and the remaining 74 variables are from organ target specific tests for neurotoxicity, nephrotoxicity, hepatocytoxicity etc. Partial least squares regression was used for the comparison with the aim to find subsets of *in vitro* tests with good predictive capability; the influence of variable reduction was studied.

The results showed that small batteries of a few *in vitro* tests give better predictive capabilities both for models based on LD50 rat (R2=0,59 and Q2=0,57) and LC50 human (R2=0,71 and Q2=0,69). The variables contributing to the best models were mainly basal cytotoxicity tests and the target organ specific tests did only improve R2 and Q2 with about 0.02, compared models based on only basal cytotoxicity tests.

The initial multivariate analysis was not sufficient to select the methods and, therefore, it was concluded that further analysis of data using different statistical approaches are needed. The analysis started in 2009 and will be performed based on the IC50 values in order to identify whether the test methods give comparable or different responses, and thus to reduce the number of tests that provide similar information (i.e. would be redundant in a testing strategy). The kinetic information generated will also be taken into account. The resulting data should allow the comparison with LD50 data and the official GHS categories. All these calculations should result in a report containing a number of test methods proposed as candidates to be included in the testing strategy. The selected assays will be challenged with a new set of chemicals during the prevalidation exercise. Finally, the analysis of the data generated during the prevalidation phase should result in the identification of the best performing strategy.

Table 1. List of in vitro *methods under evaluation in the ACuteTox project.*

Name of the test method	–
Clinical endpoint	Acute systemic toxicity; Basal cytotoxicity, hematotoxicity, kinetics, metabolism, neurotoxicity, nephrotoxicity and hepatotoxicity.
Cell (line)	WP2: Basal cytotoxicity 1. HL-60 human cell line 2. HepG2 human hepatoma cell line 3. and 6. Fa32 rat hepatoma cell line 4. Balb/3T3 mouse fibroblasts 5. Normal human keratinocytes WP4: New cell, new endpoints 7. and 8. Human peripheral blood mononuclear cells (PBMCs) 9. and 10. Human cord blood cells 11. and 14. A.704 kidney adenocarcinoma 12. and 15. HepG2 human hepatoma cell line 13. and 16. SH-SY5Y human neuroblastoma cell line WP5: Kinetics 17. Not available 18. to 20. Caco-2 intestinal cell line 21. to 23. Blood-brain barrier model 24. Not available 25. Pooled human plasma 26. Not available 27. Rat liver microsomes 28. Human liver microsomes 29. Primary rat hepatocytes 30. Cryopreserved human hepatocytes WP7.1: Neurotoxicity 31. and 32. Primary rat cerebellum granule cells (CGCs) 33. Pure enzyme 34., 35. and 37. SH-SY5Y human neuroblastoma cell line 36. Rat brain slices 38. to 40. Primary mouse cortical neurons 41. Primary mouse cerebellum granule cells (CGCs) 42. to 46. Rat re-aggregated brain cells culture 47. to 52. Primary rat cerebellum granule cells (CGCs) WP7.2: Nephrotoxicity 53. and 54. LLCPK-1 renal epithelial cell line WP6 and 7.3: Metabolism and Hepatotoxicity 55. Rat hepatocytes 56. Balb/3T3 mouse fibroblasts 57. HepG2 human hepatoma cell line.

Name of the test method	–
Method description	Known outliers of available *in vitro - in vivo* correlations are being evaluated in order to introduce further parameters that might improve the correlation, including absorption, distribution and excretion, metabolism and organ specificity. Fifty seven reference compounds were tested in the different *in vitro* systems (see Kinsner et al., 2009).
SOP	Yes (available in the database of the project).
Endpoints	WP2: Basal cytotoxicity 1. ATP content 2. and 3. Protein content (CBQCA assay) 4. Neutral red uptake Balb/3T3 mouse fibroblasts 5. Neutral red uptake Normal human keratinocytes 6. Neutral red uptake Fa32 rat hepatoma cell line WP4: New cell, new endpoints 7. Multiplexed flow cytometry analysis of cytokine release (IL-12p70, IFN-c, IL-2, IL-10, IL-8, IL-6, IL-4, IL-5, IL-1b, TNF-a, TNF-b) 8. ELISA analysis of cytokines IL-5, IFN-c and TNF-a release 9. Colony forming unit-granulocyte/macrophage (CFU-GM) 10. Colony forming unit-megakariocytes (CFU-Meg) 11. to 13. Cytomic panel for cytotoxicity screening including: Intracellular Ca2+ (Fluo-4 probe) Mitochondrial membrane potential (rhodamine123) Plasma membrane potential (DIBAC probe) Intracellular lipid content (BODIPY probe) 14. to 16. Cytomic panel for oxidative stress screening including: Intracellular peroxides Mitochondrial generation of superoxide Intracellular levels of the oxidized DNA base 8-oxo-guanine WP5: Kinetics 17. PAMPA assay 18. Intestinal absorption (permeability assay) 19. Intestinal absorption (toxicity assay – 14C-mannitol) 20. Intestinal absorption (toxicity assay – Lucifer yellow) 21. Blood–brain barrier passage (permeability assay) 22. Blood–brain barrier (toxicity assay – Lucifer yellow) 23. Blood–brain barrier (toxicity assay – 14C-sucrose) 24. Aqueous solubility 25. Plasma protein binding 26. Chromatographic hydrophobicity index (CHI)

Name of the test method	–
Endpoints	27. to 30. Metabolic stability WP7.1: Neurotoxicity 31. Alamar blue assay 32. Mitochondrial membrane potential 33. AChE inhibition 34. AChE inhibition 35. and 36. LDH leakage 37. Ca2+ overload 38. GABA-A receptor function 39. GABA uptake 40. Cell depolarization 41. Glutamate uptake 42. Gene expression (GFAP, MBP, NF-H, NF-M, PPAR-gamma, HSP-32, iNOS) 43. Enzyme inhibition/activation (ChAT, GS, AChE, LDH, 2,3-CNP) 44. Methionine uptake (protein synthesis) 45. Uridine uptake (RNA synthesis) 46. 2-Deoxyglucose uptake 47. Caspase-3 mRNA expression 48. MTT assay 49. Glutamate induced cytosolic Ca2+ increase 50. ROS production 51. LDH release 52. Microarray quantitative mRNA expression analyses of 31 genes WP7.2: Nephrotoxicity 53. Alamar blue 54. Transepithelial electrical resistance (TEER) WP6 and 7.3: Metabolism and Hepatotoxicity 55. to 57. MTT assay.
How is a positive result defined?	Dependent on the method.
How is a positive result expressed?	Dependent on the method.
Applicability	Will be known at end of the project.
Positive control	Dependent on the method.
Negative control	Dependent on the method.

Name of the test method	–
Performance	Dependent on the method.
Which R would the test method impact?	Replacement/Reduction.
How can the test be used?	As an integrated testing strategy.

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2.3 -omics, bioinformatics and computational biology

The four projects included in this chapter focus their work on predicting toxicity of substances based on the effects on organisms at a molecular level. For that purpose, they make use of methods such as genomics, proteomics, metabonomics and systems biology.

Predictomics combines culture models and a comprehensive analysis of cell effects induced by toxicants via Cytomics/Genomics/Proteomics, as well as a mechanistic knowledge of drug-induced chronic toxicity.

Sens-it-iv focuses on the understanding of the mechanisms through which chemicals and proteins induce allergy. The project's activities include metabonomics, proteomics, genomics and data management.

CarcinoGENOMICS aims to develop a series of mechanism-based *in vitro* tests based on the application of genomics technology, i.e. genome-wide transcriptomics and metabonomics.

Finally, PREDICT-IV combines the generation of metabolomic and genomic data and *in silico* modelling, to develop strategies to improve the assessment of drug safety.

Predictomics

Short-term *in vitro* assays for long-term toxicity

Contract number: LSHB-CT-2004-504761
Project type: Specific Targeted Research Project (FP6)
EC contribution: € 2 259 754
Starting date: 1 September 2004
Duration: 40 months
Website: http://www.predictomics.org
and http://www.predictomics.com

Objectives

The aim of Predictomics was to develop novel strategies for predicting chronic toxicity in the two most frequently target organs affected by drugs and xenobiotics, the liver and the kidney.

As far as the liver is concerned, a method to identify compounds that might cause liver cholestasis by means of a fluorescence-based cytometry has been designed and externally prevalidated. Fluorescent bile acid derivatives are used as probes. The method monitors real-time kinetics uptake of bile acid derivatives in fresh suspensions of hepatocytes and the impairment caused by drugs.

A second developed method is a cytometry-based multiparametric assay to identify drugs causing liver steatosis. Making use of a stable human hepatoma-derived cell line (HepG2) treated with model steatotic hepatotoxins, and image analysis after fluorimetric quantitation of the lipid content of cells, as well other cellular end-point parameters, is was possible to distinguish among weak, moderate and strong steatosic compounds.

A third developed method, made use of a genome-wide scale to identify gene products whose expression levels or post-translational features changed as a consequence of exposure to steatosic drugs. On the basis of the results obtained, a 'steatosis fingerprint' has been defined and will be exploited in the form of a DNA microarray.

Concerning renal toxicity developments, a human proximal tubule model has been established for the purposes of conducting gene expression microarrays with the Affymetrix HGU-133 plus two platforms. An interlaboratory comparison across four laboratories, using the model nephrotoxin CsA, has confirmed that the model is standardised, robust, reproducible and transferable. The predictive ability of the model

with an additional 11 nephrotoxic compounds has been investigated and a preliminary prediction model developed. A number of limitations of the model have been identified including poor performance where phase I metabolism is necessary for toxic action and an intrinsic aminoglycoside resistance. A number of marker genes have been identified which are potential markers of early response to toxicity.

Cholestasis Flow Cytometry

Objectives

It will be used primarily for fast detection by flow cytometry of putative cholestatic compounds using cell suspensions, preferentially of freshly isolated hepatocytes, but could be also applied to cells in solid support using standard fluorescence microscopy, high-content analysis systems or fluorescence spectrometry.

It will consist of a commercial kit composed of one or more fluorescent derivatives of bile acids and bile salts plus one or more positive control compounds (strong and weak cholestatic substances) and one negative control compound (a substance not causing cholestasis).

Experimental design

Freshly isolated rat hepatocytes were incubated with fluorescent derivatives of bile acids and bile salts and with cholestatic compounds (Chlorpromazine, 17α-Ethynylestradiol E, Cyclosporine, J&J01, J&J02, J&J03 and Bayer's compound that were provided by sponsors; J&J and Bayer were coded pharmaceuticals provided by Johnson & Johnson and Bayer) and Non Cholestatic compounds (Citrate and J&J04). Compounds were assayed in order to check if this method is able to classify them in putative cholestatic or non cholestatic compounds (Table 1).

Results

Results of three independent experiments (triplicates) either for non-cholestasic (Citrate and J&J04) or cholestasic compounds (J&J01, J&J02, J&J03, Bayer, Cyclosporine, Chlorpromazine and 17α-Ethynylestradiol E) showed that this assay was able to classify tested compounds according their impairment of drug uptake. Therefore, this study has been found robust and reproducible as it is shown in the results obtained with compounds previously tested by this method in other laboratories.

Next steps

Now that the project is finished, further experiments are needed in order to be offered to the pharmaceutical companies as an assay for testing potential cholestasic compounds.

Publications

1. Photophysical characterization and flow cytometry applications of cholylamidofluorescein, a fluorescent bile acid scaffold. Rohacova J, Marin ML, Martínez-Romero A, O'Connor JE, Gomez-Lechon MJ, Donato MT, Castell JV, Miranda MA. *Photochem Photobiol Sci.* 7:860-866. (2008).

2. Fluorescent Benzofurazan-Cholic Acid Conjugates for in vitro Assessment of Bile Acid Uptake and Its Modulation by Drugs.Rohacova J, Marín ML, Martinez-Romero A, Diaz L, O'Connor JE, Gomez-Lechon MJ, Donato MT, Castell JV, Miranda MA. *ChemMedChem.* 4:466-472 (2009).

Table 1

Name of the test method	Assay for drug-induced liver Cholestasis by flow cytometry
Clinical endpoint	Screening of drug-induced Cholestasis.
Cell (line)	Freshly isolated rat hepatocytes.
Method description	Incubations are performed in propilene tubes containing 500 µl of hepatocyte suspension ($5x10^5$ viable cells/ml). Compounds are concomitantly administered with the bile-acid fluorescent derivative by diluting both stock solutions 1:50 in the cell suspension and incubated in a water bath at 37ºC for 15 min. Five minutes before the incubation ending, propidium iodide, a marker of cell viability, is added at a final concentration of 0.0025 mg/ml. Finally, cell suspension is analysed with a flow cytometer that allows discrimination in parallel between alive and dead cells and analysis of the uptake of the bile acid derivatives only in the former ones.
SOP	Yes.
Endpoints	Quantification of fluorescent bile acid transport rates inside cells by means of flow cytometry.
How is a positive result defined?	Compounds are considered to be cholestasic when fluorescence values are over control value.
How is a positive result expressed?	% of fluorescence over control (untreated cells).
Applicability	Drug screening of cholestasis in drug development.
Positive control	Chlorpromazine, 17α-Ethynylestradiol E, Cyclosporine, J&J01, J&J02, J&J03 and Bayer's compound.
Negative control	Citrate and J&J04.
Performance	Sensitivity, positive and negative predictive value.
Can the test method be used in a regulatory safety context?	Yes.
Which R would the test method impact?	Refinement.
How can the test be used?	Either as a stand-alone method for screening purposes or as part of an testing strategy for drug development.

Steatosis Flow Cytometry

Objectives

Development of an assay for the fast detection of putative steatosic compounds by flow cytometry using a stable human hepatoma-derived cell line (HepG2 cells).

Experimental design

HepG2 cells were seeded on 24 well/plates in a monolayer confluency never higher than 75%. Attached cells were fat-overloaded by incubating monolayers for a 12-hour period with a 62 µM free fatty acid (FFA) mixture (Palmitate and Oleate) diluted in culture medium supplemented with 5% fetal bovine serum (FBS) and 30% bovine serum albumin (BSA). At the end of the fat-overloading period, test compounds diluted in lipid-free medium were administered to HepG2 cells and incubation run on for an additional 24-h period.

To screen for drug-induced liver steatosis, HepG2 cells were incubated with steatotic compounds (Tetracycline, Sodium Valproate, Amiodarone, J&J01, J&J02 and J&J03) and non steatotic compounds (Citrate and J&J04). J&J are coded pharmaceuticals provided by the sponsor Johnson & Johnson.

Tetracycline and Citrate were used as positive and negative controls of fat accumulation, respectively.

BODIPY®493/503 (4,4-difluoro-3a, 4a-diaza-s-indacene) is a fluorophore that is intrinsically lipophilic and mimics the properties of natural lipids. This dye has potential applications as stain for neutral lipids and as a tracer for oils and other non-polar lipids. On this basis, BODIPY®493/503 was used to analyse fat accumulation in the HepG2 cell line (SOP-LIPID BODIPY-d02). To do so, non-treated and drug-treated HepG2 cells were used to evaluate drug effects on cell uptake of fatty acids, using a fluorescent fatty acid derivative and flow cytometry. The fatty acid analogue has an emission wavelength of 510 nm, so transport inside the cells can be detected and quantified by measuring the increase of green fluorescence in cells after the exposure to these fluorophore. In addition, the flow cytometry assay allows the discrimination of the mitochondrial membrane potential in parallel samples, an indicator of cell toxicity, by means of Tetramethyl rhodamine methyl ester (TMRM), a fluorochrome with an emission wavelength of 580 nm.

Alive and dead cells could be also differentiated by using orange-fluorescence propidium iodide, that emitts at 620 nm. This fluorescent marker allows the analysis of the uptake of both fluorochromes only in viable cells (Table 2).

Results

Results of three independent experiments (triplicates) either for non-steatosic (Citrate and J&J04) or steatosic compounds (J&J01, J&J02, J&J03, Tetracycline, Amiodarone and Sodium Valproate) showed that this assay was able to classify most of compounds tested in this assay according to their steatosic effect. Although more experiments should be performed to really show the robustness of this method.

Next steps

Now that the project is finished, further experiments are needed. The assay will be offered to the pharmaceutical companies as a service for testing potential steatosic compounds.

Table 2

Name of the test method	Multiparametric assay for drug-induced steatosis in HepG2 cells by flow cytometry
Clinical endpoint	Screening of drug-induced steatosis.
Cell (line)	HepG2 cells.
Method description	HepG2 cells are seeded in 24 well/plates. When they reach 75% confluency, cells are exposed to the compounds for 24-h, period after which monolayers are washed with PBS and finally detached by trypsin/EDTA (0.05%/0.02%) treatment at 37ºC. After centrifugation, cell pellet is resuspended in fluorochrome-free medium. Subsequently, fluorochromes are added and incubated 30 min prior to analysis by flow cytometry multiparametric analysis.
SOP	Yes.
Endpoints	Fatty acid accumulation and alteration of mitochondrial membrane potential are the parameters chosen to evaluate steatosis and cell toxicity, respectively.
How is a positive result defined?	Compounds are considered to be steatosic when fluorescence values are over control value.
How is a positive result expressed?	% of fluorescence over control (untreated cells).
Applicability	Drug screening of steatosis in drug development.
Positive control	Tetracycline, Sodium Valproate, Amiodarone, J&J01, J&J02 and J&J03.
Negative control	Citrate and J&J04.
Performance	Sensitivity, positive and negative predictive value.
Can the test method be used in a regulatory safety context?	Yes.
Which R would the test method impact?	Refinement.
How can the test be used?	Either as a stand-alone method for screening purposes or as part of an testing strategy for drug development.

SteatoChip

Objectives

The objective was to identify genes that undergo changes in their expression levels as a response to induction of steatosis in a liver cell-line and generate a steatosis fingerprint in the form of a DNA microarray.

Experimental design

HepG2 were treated with two-six different steatotic compounds (Amiodarone, Valproate, Tetracycline, Free Fatty Acids, J&J 2, J&J 3) and a non-steatotic compound (J&J 4) J&JPred2, J&JPred3 and J&JPred4 were coded pharmaceuticals provided by Johnson & Johnson. The expression levels were compared to those obtained from untreated cells (Solvent controls) using Affymetrixs U133A microarrays for transcriptomics (Figure 1 and Table 3).

Results

A concluding observation from the comparison of the different treatments is that there was a great variation in response to the various compounds: *i*) most treatments did not give rise to significant changes in expression levels which resulted in insufficient number of differential expressed genes, *ii*) the majority of the differential transcripts were unique for each treatment with only a few genes shared by more than two treatments.

Due to these results, we defined the steatotic specific genes as those transcripts that had a FoldChange greater than two (FC>2) and present in at least two of the steatosis treatments and at the same time absent in the non-steatosis treatment (J&JPred4). This resulted in 30 induced genes (31 probe sets) and 31 repressed genes (36 probesets) that together constitute the 'Steatotic Finger-Print'. It should be noted that three genes fulfilling the above criteria were present in more than two of the treatments.

Due to the low number of 'Finger-Print' genes, gene ontology analysis did not result in any statistically significant pathways. A manual examination of the gene list could however give interesting information with respect to the cellular response to treatments with steatotic compounds.

Next steps

Now that the project is finished, further improvements are needed in order to make exploitable the SteatoChip in the form of a DNA microarray. These specific DNA transcripts should be refined before exploitation: further research will be required on a broader selection of compounds where cooperation with pharma companies would be a must to continue the development of such a device. Another possible use of the device would be screening drugs in early stages of development.

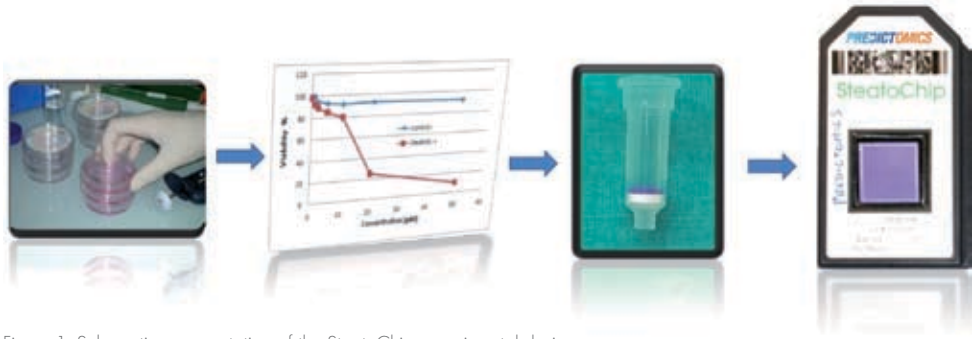

Figure 1. Schematic representation of the SteatoChip experimental design.

Table 3

Name of the test method	SteatoChip
Clinical endpoint	Screening of drug-induced steatosis.
Cell (line)	HepG 2.
Method description	Incubation of selected model compound in HepG2 Cells, mRNA extraction and perform Affimetrixs analysis.
SOP	No.
Endpoints	Gene expression changes; Affimetrixs.
How is a positive result defined?	Hierarchical clustering groups. Gene expression Changes.
How is a positive result expressed?	Fold Change.
Applicability	Screening of putative steatotic compounds.
Positive control	Steatotic compounds (Amiodarone, Valproate, Tetracycline, Free Fatty Acids, J&J 2, J&J 3).
Negative control	Non-steatotic compound (J&JPred2, J&JPred3 and J&JPred4).
Performance	Sensitivity, positive and negative predictive value.
Can the test method be used in a regulatory safety context?	Yes.
Which R would the test method impact?	Refinement.
How can the test be used?	As part of an alternative/integrated testing strategy.

Nephrotoxicity

Objectives

A major focus of the Predictomics project was to investigate the use of emerging techniques such as transcriptomics in the *in vitro* hepato- and nephro- toxicity fields. As part of this task a major question arose regarding the reproducibility of transcriptomic data from different laboratories.

Genome-wide DNA microarrays allow the relative quantification of the entire RNA population. Since it is expected that certain modes of toxicity will lead to different gene expression patterns, this technique promises both to improve toxicity prediction and also to uncover novel mechanisms of toxicity and toxicotolerance. However, if DNA microarrays are to become part of *in vitro* toxicity testing regimes, it must first be assessed whether RNA profiles after a chemical or pharmaceutical stimulus from different laboratories are comparable. Therefore, we designed an interlaboratory study comprised of four European laboratories, with expertise in *in vitro* nephrotoxicity, to investigate the effect of cyclosporine A (CsA) on global gene expression in cultured human proximal tubular cells.

Experimental Design

A major focus of this study was to standardise cell culture and treatment regimes across the participating laboratories. The human proximal tubule cell line, HK-2 was newly purchased from ATCC and distributed to all laboratories. Cells were cultured in serum free hormonally defined DMEM-F12. A non-cytotoxic concentration of CsA (5 μM) was used for the transcriptomic profiling at two exposure time-points (12h and 48h) (Table 4).

Results

The results of the genome wide transcriptomic profiling revealed that one laboratory clustered from the other three laboratories. However, this laboratory had used an additional trypsinisation step before RNA isolation and thus the genes responsible for this clustering were removed from further analysis. Once this was done, all laboratories showed a similar pattern of gene expression.

There was a large alteration in gene expression in control cells due to time alone (12h control vs 48h control). On further analysis, this was deemed to be due to medium exhaustion. Medium glucose decreased and medium lactate increased continuously over culture time. IL-6, as an example of an autocrine factor, increased over time in culture. And LDH, as an example of a cytosolic enzyme which is released upon cell damage, was also increased over time in culture.

To unravel the effects of CsA exposure, we expressed the values as fold over time matched control. Across all four laboratories, a robust alteration of gene expression by CsA was demonstrated. CsA responsive genes were involved in cell cycle, the p53 pathway and Wnt signalling.

This study demonstrates that transcriptomic profile is a robust tool for investigating the effects of compounds on cells in culture. Additionally this technique is unbiased and no previous knowledge of the effects of the compound is required.

The study was recently published in *Toxicology In Vitro* (Jennings et al. *Inter-laboratory comparison of human renal proximal tubule (HK-2) transcriptome alterations due to Cyclosporine A exposure and medium exhaustion.* Toxicology in Vitro, 23 pp 486-499).

Table 4

Name of the test method	Nephrotoxicity
Clinical endpoint	-
Cell (line)	Human renal proximal tubule (HK-2).
Method description	RNA extraction and Affymetrix DNA array.
SOP	Internal.
Endpoints	RNA profiling.
How is a positive result defined?	Statistical.
How is a positive result expressed?	Fold change.
Applicability	Toxicity pathways.
Positive control	CsA (5 μM).
Negative control	Ethanol (vehicle).
Performance	Robust.
Can the test method be used in a regulatory safety context?	Not yet.
Which R would the test method impact?	Reduction.
How can the test be used?	As part of an alternative/integrated testing strategy.

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Sens-it-iv

Novel testing strategies for *in vitro* assessment of allergens

Contract number: LSHB-CT-2006-018681
Project type: Integrated Project (FP6)
EC contribution: € 10 999 700
Starting date: 1 October 2005
Duration: 60 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-legislation on chemicals (REACH) is expected to consume millions of animals per year. Conversely, several EU legislations call for significant reductions or even a complete ban on animal testing (i.e. for cosmetics and cosmetic ingredients since 11.03.2009).

Therefore, 28 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 industry.

Objectives

A. Scientific objectives (Science Module)

The Science Module aims to acquire a solid understanding of the processes occurring *in vivo* tissue challenged by a potential sensitiser and to compare this with molecular indicators on the cells involved in these reactions. Based on this understanding, it intends to develop assay systems that model sensitisation, rather than irritation and toxicity of chemicals and proteins.

1. Description of the *ex vivo* phenotypic changes of human lung epithelial cells (EC), antigen presenting dendritic cells (DC) and effector T cells using functional genomics, proteomics and immunohistology before and after challenge of lung tissue slices.

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

3. Description of bio-activation and hapten-formation, as well as of the chemical structures and peptide sequences involved in hapten-formation, using advanced matabonomic and proteomic technologies.

B. Technological objectives (Technology Module)

The aim of the technology module is to evaluate and further develop the resulting *in vivo*-like tests models, and to establish *in vitro* assays ready for prevalidation and approval by ECVAM, the European Center for Validation of Alternative Methods.

1. Set-up of an inductive database for the acquired scientific data, including al available literature information to allow queries for data patterns and predictive models.

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

3. Refinement and optimisation of these assays for prevalidation.

C. Dissemination objectives (Dissemination and Technology Transfer Module)

The aim of this Module is to ensure awareness (inside as well as outside the consortium) of the activities and results of Sens-it-iv, and of progress made by the consortium towards the ultimate objective: 'Novel testing strategies for *in vitro* assessment of allergens'.

1. Set-up and maintenance of an internal and external website.

2. Production of monthly newsletters.

3. Assuring proper documentation of results from and knowledge acquired by the consortium.

4. Publications in non-scientific journals and magazines targeting laymen.

5. Organisation of two training courses.

Experimental design

In order to meet the objectives, the activities of the Sens-it-iv project focused on three major areas. Each Module is organised in work packages (WPs), each of them coordinating the various disciplines addressing the specific tasks and objectives (Figure 1).

A. Science Module (Month 0-36) – Basic Research

The Science Module is about implementing existing and acquired knowledge and understanding of how the anticipated key players in sensitisation (e.g. EC, DC and T cells) exist and interact in normal tissue, and how these interactions are disturbed *in vivo* when tissue is challenged by a potential sensitiser.

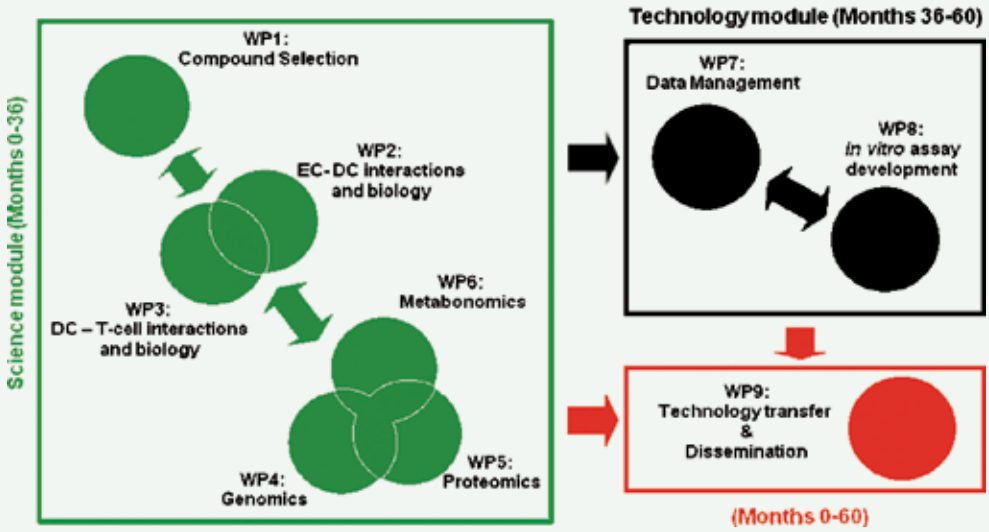

Figure 1. Overview on the project activities and WP1-9 interactions.

While sufficient knowledge is available with respect to the skin, the cellular and molecular events occurring *in vivo* in the lung are poorly described. To fill this gap, the precision cut lung slice (PCLS) technology (*ex vivo*) is implemented by WP2 to provide the project with the *in vivo* human information required for the development of mechanistically relevant *in vitro* test systems for assessing respiratory sensitisation.

The available and acquired information is used by WP2 to identify the most *in vivo*-like EC and DC, as well as the cell culture conditions supporting the *in vivo*-like phenotype of these cells and the *in vivo*-like cell-cell interactions. Human effector (Teff) and regulatory (Treg) T cells, and NK cells, which have been recognised as playing an important role in sensitisation, are characterised by WP3.

The relevant cells and/or cell lines, and cell culture conditions, are used by WP2 and WP3 to develop assay systems (prototypes) that model sensitisation, rather than irritation and toxicity of chemicals and proteins.

In order to make assay development possible, a list of well-characterised tutorial chemicals is required (WP1).

The Science Module also includes three WPs, providing the respective technologies: Genomics (WP4), Proteomics (WP5) and Metabonomics (WP6).

WP4 and WP5 support WP2 and WP3 in their efforts to describe, at molecular level, the response signatures that are involved in processes occurring when tissue and cells are challenged by a potential sensitiser, irritant or inert compound.

Finally, WP6 is to increase the understanding of the molecular basis of the interactions between chemical sensitisers, and extracellular and intracellular protein, as well as of the biological effects of allergens. This understanding will give supplementary information for any validation procedure of test development.

B. Technology Module
(Month 36-60) – Applied Research

The Technology Module is to provide the tool allowing data management and usages (WP7) and to further develop and refine selected assays, and prepare them for prevalidation (WP8).

WP7 maintains the internal database that can capture the huge amount of data and information that are produced by the WPs in the Science Module, and assures that these data and information are readily available to the partners.

Measures are taken to assure that the Sens-it-iv database will continue to exist after closure of the project, including incorporation into the FP7 project OpenTox and data sharing with the ToxCast initiative.

The selection of assays for the Technology Module (WP8) is, according to a standard operation procedure (SOP) provided by WP1. This procedure is based upon the criteria used internally by ECVAM. Annex 1 lists the critical issues to be covered.

Although not fully complying with all the selection criteria, the PCLS is now considered as a test system for respiratory sensitisation. This consideration is based upon the promising results obtained with this test as well as upon the lack of any alternative (primary cell or cell line). The potentials and limitations of this approach are currently assessed by WP2.

To date, seven potential cell-based assays are identified and are currently evaluated, further developed and refined.

1. **Three DC-based submerged assays (Annex 2-4):** The three DC-based assays are compared and their potential to discriminate sensitisers from non-sensitisers is assessed on the 21 tutorial chemicals.

2. **An assay based upon the human keratinocyte cell line NCTC 2544 (Annex 5):** The test is standardised and harmonised between the Sens-it-iv laboratories for further evaluation with a more extended panel of chemical sensitisers, irritants and controls.

3. **The EC-based potency tests (Annex 6):** The prototype test contains an Epidermal Equivalent Skin model developed by Sens-it-iv. For applicability reasons, the technology is transferred to a commercially available skin model. The test is standardised and harmonised between the Sens-it-iv laboratories for further evaluation with a more extended panel of chemical sensitisers, irritants and controls.

4. **The DC-migration tests (Annex 7):** The test is refined to improve its industrial applicability. It is standardised and harmonised between the Sens-it-iv laboratories for further evaluation with a more extended panel of chemical sensitisers, irritants and controls.

5. **A T cell priming assay (Annex 8):** The test is refined to improve its industrial applicability. It is standardised and harmonised between the Sens-it-iv laboratories for further evaluation with a more extended panel of chemical sensitisers, irritants and controls.

WP4 and WP5 activities related to biomarker identification are extended by six months. The primary objective is to provide WP8 (Technology Module) with novel (but applicable) EC, DC, EC-DC and T cell response signatures that are specific for sensitisation.

C. Dissemination and Technology Transfer Module (Month 0-60)

The scientific and technological results generated by the various WPs are disseminated using the structures and tool established during the project period.

Technology transfer to relevant stakeholders (exploitation) and activities geared towards education and training are initiated and intensified.

Results

Preliminary results

1. **The list of chemicals was finalised:** The list of 21 tutorial chemicals was expanded to 29. These 29 compounds are deemed sufficient by the consortium for further development and standardisation of the tests under evaluation (Table 1).

2. **Lung sensitisation: links from *in vivo* to *in vitro*:** The precision-cut-lung-slices (PCLS) technology, originally using murine, and now lung material, identified DC of the alveolar compartment as major target cells of inhaled substances. Comparative *in vitro* co-cultures revealed essential requirements for direct EC-DC interaction.

3. ***In vivo*-like EC and DC primary cells or established cell lines:** Catalogues of primary EC and DC, as well as cell lines were established in order to help identify the most *in vivo*-like and readily available EC and DC cell. The myeloid DC line MUTZ-3 exhibited the most *in vivo*-like properties. For skin EC, work is focusing on primary human keratinocytes and the NCTC2544 cell line, while a candidate for lung EC remains to be defined.

4. **EC-DC co-culture systems:** EC-DC cross talk is addressed in various culture systems. The advantage of the 2-compartment (airlifted) co-culture model is that it has incorporated the EC-mediated barrier function and allows for topical application even of chemicals insoluble in water. Whether, in addition to this, there is an added value above mono-cultures still has to be determined. Since sensitiser-specific readouts are observed in non-separated EC-DC co-cultures, direct EC-DC contact might be essential. Genomic, proteomic

and kinomic analysis, and CD-antibody array studies are being used to establish 'signatures' of activation markers.

5. ***In vitro* induction of DC migration:** Langerhans cells (LC) or the corresponding MUTZ-3 derived MUTZ-LC cells migrate towards the recombinant chemokines CXCL12 or CCL5 if activated by sensitisers or irritants, respectively. The tutorial chemicals are under investigation in a dual-chamber test system.

6. **The role of T cells in sensitisation:** Proliferative *in vitro* stimulation of naïve human T cells with chemically modified DC can be indicative of sensitisation for allergic contact dermatitis (ACD). Effective methods have been developed to expand allergen specific T cells from patient blood by several orders of magnitude.

7. **Innate immunity essential for contact allergy:** The involvement of innate immune responses in the induction of ACD is indicated by the infiltration of natural killer (NK) cells into allergen-exposed skin, and the essential requirement of Toll-like receptors 2 (TLR-2) and 4. Both findings open ways to define additional targets for the development of *in vitro* skin sensitisation assays.

8. **The impact of metabolomics:** One pre-requisite of the ability of chemicals to sensitise, is their potential to bind (usually covalently) to proteins. Many allergens, however, are not protein-reactive *per se*, but require enzymatic transformation into reactive metabolites. Present work aims at increasing the expression/activity of metabolizing enzymes in *in vitro* assays, and at enhancing the concentration of intracellular chemical by promoting cellular influx and/or inhibiting efflux pathways.

9. **Marker identification a key for assay development:** Identifying markers for each of the key events of allergic sensitisation is the prerequisite for the development of any *in vitro* assay. Sens-it-iv employs gene expressing profiling, proteomics (gel or liquid chromatography with tandem-mass-spectroscopy), CD-antibody arrays and kinomics to define new markers or even complex marker signatures to differentiate skin and lung sensitisers from mere irritants.

Table 1. Final list of chemicals.

Respiratory	Skin	Controls
Diphenylmethane diisocyanate (MDI)	2,4 dinitrochlorobenzene (DNCB)	Sodium lauryl sulphate (SLS)
Trimellitic anhydride (TMA)	Cinnamaldehyde (CIN)	Salicylic acid (SA)
Ammonium hexachloroplatinate (AHPt)	Tetramethyl thiuram disulfide (TMTD)	Phenol (Ph)
Hexamethylene diisocyanate (HDI)	Resorcinol	Glycerol
Maleic anhydride (MA)	Oxazolone	Lactic acid
Glutaraldehyde (GA)	Glyoxal	Chlorobenzene
	2-mercaptobenzothiazole (MBT)	P-hydroxybenzoic acid
	2-bromo-2-(bromomethyl) glutaronitrile (BBGN)	Benzaldehyde
	4-nitrobenzylbromide (NBB)	Diethyl phtalate
	Pre/pro-haptens	Octanoic acid
	Isoeugenol	
	Eugenol	
	Cinnamic alcohol (CA)	
	Paraphenylendiamine	

Next steps

The major tasks for the remaining time of the project concentrate on:

1. selection of methods with potential of being developed into reliable assays,
2. evaluation, further development and refinement of these assays,
3. decisions on which parts of the basic R&D work should be continued.

As yet, five types of assays (seven in total) have been identified and are currently being evaluated, further developed and refined (See Experimental Design).

In addition, Sens-it-iv continues to support more basic-research to further assess the usefulness of the PCLS as a test system for assessing respiratory sensitisation.

Finally, an additional six months was given to biomarker identification.

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Annex 1

Name of the test method	Could be an internal code/reference
Clinical endpoint	E.g. sensitisation, carcinogenesis, repeated dose, etc.
Cell (line)	E.g. primary human bronchial cells, Caco2, etc.
Method description	Summary of how the test is performed.
SOP	Yes/No.
Endpoints	E.g. CD86, IL-8, etc. or combinations of markers (should include detection methods (e.g. PCR, FACS, etc.))
How is a positive result defined?	Criteria used to classify positive (and negative) results, e.g. cut-off values, profile of relevant markers, etc.
How is a positive result expressed?	E.g. fold change, absolute values, etc.
Applicability	E.g. compounds tested, definition of the class(es) of compounds.
Positive control	What was used?
Negative control	What was used?
Performance	Sensitivity, specificity, accuracy, positive and negative predictive value.
Can the test method be used in a regulatory safety context?	Yes/No. If yes, which EU or other regulatory requirements would help to address?
Which R would the test method impact?	Replacement/Reduction/Refinement.
How can the test be used?	As a stand-alone method or as part of an alternative/integrated testing strategy?
Other important remarks?	Any additional relevant information.

Annex 2

Name of the test method	MUTZ-3 test (CD86, IL-8)
Clinical endpoint	Sensitisation.
Cell (line)	MUTZ-3.
Method description	It concerns a DC-based submerged assay. Cell cultures are exposed to 4 concentrations of chemical. The concentrations were defined based upon the relative cell viability (rCV) as determined using the propidium iodide (PI) method: concentration (C) 1 = 75% rCV, C 2 = 75% C 1, C 3 = 50% C 1, C 4 = 50% C 2. Incubation was for 24 hrs.
SOP	Yes.
Endpoints	CD86, IL-8.
How is a positive result defined?	Increase in CD86+ cells and increased IL-8 production.
How is a positive result expressed?	CD86: the stimulation index consists of 2 variables (CD86 expression intensity and CD86+ cells (%) IL-8: > 2x.
Applicability	Skin sensitisers: 1,4-dinitrochlorobenzene (DNCB), cinnamaldehyde; Irritants: sodium lauryl sulphate (SLS), salicylic acid.
Positive control	DNCB.
Negative control	Medium.
Performance	Only DNCB and CIN stimulated CD86 and IL-8 production. To be determined using an extended list of chemicals.
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Replacement.
How can the test be used?	Integrated testing strategy.
Other important remarks?	Extensive standardisation and harmonisation between the Sens-it-iv laboratories was successfully concluded. The test is currently under evaluation with an extended panel of chemical sensitisers and irritants.

Annex 3

Name of the test method	U937 test (CD86, IL-8)
Clinical endpoint	Sensitisation.
Cell (line)	U937.
Method description	It concerns a DC-based submerged assay. Cell cultures are exposed to 4 concentrations of chemical. The concentrations were defined based upon the relative cell viability (rCV) as determined using the propidium iodide (PI) method: concentration (C) 1 = 75% rCV, C 2 = 75% C 1, C 3 = 50% C 1, C 4 = 50% C 2. Incubation was for 24 hrs.
SOP	Yes.
Endpoints	CD86, IL-8.
How is a positive result defined?	Increase in CD86+ cells and increased IL-8 production.
How is a positive result expressed?	CD86: the stimulation index consists of 2 variables (CD86 expression intensity and CD86+ cells (%) IL-8: > 2x.
Applicability	Skin sensitisers: 1,4-dinitrochlorobenzene (DNCB), cinnamaldehyde; Irritants: sodium lauryl sulphate (SLS), salicylic acid.
Positive control	DNCB.
Negative control	Medium.
Performance	Only DNCB and CIN stimulated CD86 and IL-8 production. To be determined using an extended list of chemicals.
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Replacement.
How can the test be used?	Integrated testing strategy.
Other important remarks?	Extensive standardisation and harmonisation between the Sens-it-iv laboratories was successfully concluded. The test is currently under evaluation with an extended panel of chemical sensitisers and irritants.

Annex 4

Name of the test method	THP-1 test (CD86, IL-8)
Clinical endpoint	Sensitisation.
Cell (line)	THP-1.
Method description	It concerns a DC-based submerged assay. Cell cultures are exposed to 4 concentrations of chemical. The concentrations were defined based upon the relative cell viability (rCV) as determined using the propidium iodide (PI) method: concentration (C) 1 = 75% rCV, C 2 = 75% C 1, C 3 = 50% C 1, C 4 = 50% C 2. Incubation was for 24 hrs.
SOP	Yes.
Endpoints	CD86, IL-8.
How is a positive result defined?	Increase in CD86+ cells and increased IL-8 production.
How is a positive result expressed?	CD86: the stimulation index consists of 2 variables (CD86 expression intensity and CD86+ cells (%) IL-8: > 2x.
Applicability	Skin sensitisers: 1,4-dinitrochlorobenzene (DNCB), cinnamaldehyde; Irritants: sodium lauryl sulphate (SLS), salicylic acid.
Positive control	DNCB.
Negative control	Medium.
Performance	Only DNCB and CIN stimulated CD86 expression. However, also SLS triggered IL-8 production. To be determined using an extended list of chemicals.
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Replacement.
How can the test be used?	Integrated testing strategy.
Other important remarks?	Extensive standardisation and harmonisation between the Sens-it-iv laboratories was successfully concluded. The test is currently under evaluation with an extended panel of chemical sensitisers and irritants.

Annex 5

Name of the test method	NCTC 2544 test (IL-18)
Clinical endpoint	Sensitisation.
Cell (line)	NCTC 2544.
Method description	It concerns a human keratinocyte-based submerged assay. Cell cultures of the cell line NCTC2544 are exposed to 3 concentrations of chemical. The concentrations were defined based upon the relative cell viability (rCV) as determined using the MTT method: concentration (C) 1 = 75% rCV, C 2 = 50% C 1, C 3 = 50% C 2, C 4 = 50% C 3. Incubation was for 24 hrs.
SOP	Yes.
Endpoints	IL-18.
How is a positive result defined?	Increased IL-18 production (intracellular).
How is a positive result expressed?	IL-18: > 2x.
Applicability	Skin sensitisers: 1,4-dinitrochlorobenzene (DNCB), cinnamaldehyde, cinnamyl alcohol, eugenol, glyoxal, isoeugenol; resorcinol, p-phenyldiamine (PPD), tetramethylthiuram disulfide (TMTD), 2-mercaptobenzothiazole, 4-nitrobenzylbromide Respiratory sensitisers: ammonium hexachloroplatinate (HClPt), 2-phenylmethane diisocyanate (MDI), trimellitic anhydride (TMA); Irritants: phenol, lactic acid, sodium lauryl sulphate (SLS), salicylic acid; Negative: glycerol.
Positive control	DNCB.
Negative control	Medium.
Performance	Only skin sensitisers were positive, while respiratory sensitisers and irritants did not induced intracellular IL-18 production.
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Replacement.
How can the test be used?	Integrated testing strategy.
Other important remarks?	Extensive standardisation and harmonisation between the Sens-it-iv laboratories is in progress. The test is currently under evaluation with a more extended panel of chemical sensitisers and irritants.

Annex 6

Name of the test method	EC-potency test (Epidermal Equivalent (EE) Model)
Clinical endpoint	Sensitisation.
Cell (line)	Primary keratinocytes.
Method description	It concerns a 3-dimensional (airlifted) assay involving human primary keratinocytes. The reconstituted skin is exposed to a dilution of the selected chemicals, starting from the toxic levels. Incubation was for 24 hrs.
SOP	Yes, but to be resubmitted after transfer (See Remarks).
Endpoints	Cell viability as determined by the MTT assay.
How is a positive result defined?	The chemical concentration corresponding to the EC50, i.e. resulting in 50% loss of cell viability.
How is a positive result expressed?	µM corresponding to EC50.
Applicability	Skin sensitisers: 1,4-dinitrochlorobenzene (DNCB), cinnamaldehyde, cinnamyl alcohol, tetramethylthiuram disulfide (TMTD); Respiratory sensitisers: ammonium hexachloroplatinate (HClPt), 2-phenylmethane diisocyanate (MDI), trimellitic anhydride (TMA); Irritants: phenol, sodium lauryl sulphate (SLS), salicylic acid.
Positive control	DNCB.
Negative control	Medium.
Performance	The EC50 release is related to the sensitising potency of the chemicals as determined by the local lymphnode assay (LLNA). The respiratory sensitisers could not be assessed (no toxicity observed).
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Replacement.
How can the test be used?	Integrated testing strategy, e.g. in combination with a DC-based assay (Annex 2-4) or the NCTC 2544-based assay (Annex 5).
Other important remarks?	Transfer from the EE to a commercially available skin model is in progress (for the sake of industrial applicability). Extensive standardisation and harmonisation between the Sens-it-iv laboratories is planned. The test is to be evaluated with a more extended panel of chemical sensitisers and irritants.

Annex 7

Name of the test method	DC-migration assay
Clinical endpoint	Sensitisation.
Cell (line)	MUTZ-3 derived LC.
Method description	It concerns a two compartment test where the compartments are separated by a membrane allowing cellular migration. The upper compartment contains the cells, while the lower compartment contains recombinant chemokines (CXCL12 or CCL5). Exposure of the CSFE-labeled MUTZ-LC to sensitisers results in a cell migration towards CXCL12, while exposure to irritants results in a cell migration towards CCL5. Incubation is for 16 hrs.
SOP	Yes.
Endpoints	Number of MUTZ-LC cells (as determined by fluorescence measurements) in the lower compartment.
How is a positive result defined?	Sensitiser: CXCL12/CCL5 > 1.0 Non-sensitiser: CXCL12/CCL5 < 1.0.
How is a positive result expressed?	Sensitiser: CXCL12/CCL5 > 1.0 Non-sensitiser: CXCL12/CCL5 < 1.0.
Applicability	Skin sensitisers: 1,4-dinitrochlorobenzene (DNCB), cinnamaldehyde, cinnamyl alcohol, tetramethylthiuram disulfide (TMTD); Respiratory sensitisers: ammonium hexachloroplatinate (HClPt), 2-phenylmethane diisocyanate (MDI), trimellitic anhydride (TMA); Irritants: phenol, sodium lauryl sulphate (SLS), salicylic acid.
Positive control	DNCB.
Negative control	Medium.
Performance	Clear discrimination between sensitisers and non-sensitisers.
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Replacement.
How can the test be used?	Integrated testing strategy.
Other important remarks?	Extensive standardisation and harmonisation between the Sens-it-iv laboratories is planned. The test is to be evaluated with a more extended panel of chemical sensitisers and irritants.

Annex 8

Name of the test method	T-cell priming assay
Clinical endpoint	Sensitisation.
Cell (line)	Human T-cell clones.
Method description	The assay consists of two steps. The first step is a T cell amplification step involving limiting dilution of the T cells and polyclonal stimulation, resulting in an up to 5000x enrichment of specific T cells. The second step is the actual T cell priming assay where T cell clones are stimulated in the presence of autologous DC and compound for 72 hrs. Chemicals are introduced with and without coupling to human serum albumin.
SOP	Yes.
Endpoints	Antigen-specific IL-2 production.
How is a positive result defined?	–
How is a positive result expressed?	–
Applicability	Skin sensitisers: 1,4-dinitrochlorobenzene (DNCB), cinnamaldehyde, cinnamyl alcohol, tetramethylthiuram disulfide (TMTD); Respiratory sensitisers: industrial enzymes and environmental allergens.
Positive control	DNCB.
Negative control	Medium.
Performance	To be determined.
Can the test method be used in a regulatory safety context?	No.
Which R would the test method impact?	Replacement.
How can the test be used?	Integrated testing strategy.
Other important remarks?	Extensive standardisation and harmonisation between the Sens-it-iv laboratories is planned. The test is to be evaluated with a more extended panel of chemical sensitisers and irritants.

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

Objectives

The carcinoGENOMICS project aims to develop *in vitro* methods to test the carcinogenic properties of compounds as an alternative to the chronic rodent bioassays that assess chemical genotoxicity and carcinogenicity. The major goal is to develop a series of mechanism-based *in vitro* tests that are representative of various modes of carcinogenic action for a number of major target organs for carcinogenic action e.g. liver, lungs, and kidneys. It has also the objective of building an iterative *in silico* model of chemical carcinogenesis. This will enable the efficient assessment of high numbers of compounds for genotoxicity and carcinogenicity as required under the REACH (Registration, Evaluation and Authorisation of Chemical) initiative, while reducing *in vivo* testing.

In developing toxicogenomics-based tests for chemical safety, carcinoGENOMICS addresses a crucial area within the LifeSciHealth Priority, namely 'the development of new *in vitro* tests to replace animal experimentation'. With reference to the Three R Principle (Replace, Reduce, Refine), as highlighted in the LifeSciHealth Priority, the project is directed towards replacing chronic rodent bioassays for assessing chemical genotoxicity and carcinogenicity.

The project also relates to the overall aim of the LifeSciHealth Priority, namely 'To build on the sequencing of the human genome and many other genomes with the result of improving human health and to stimulate industrial and economic activity'. This may be translated into practical terms by improved exposure standards in EU chemical policymaking, thereby reducing human health risks, and upgrading quality of life and environment at the European level.

Experimental design

The novel assays will be based on the application of genomics technologies (i.e. genome-wide transcriptomics and metabonomics) 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 a holistic understanding of systems biology, and then used to build an iterative *in silico* model of chemical carcinogenesis.

carcinoGENOMICS is structured in work packages (WPs) as shown in Figure 1.

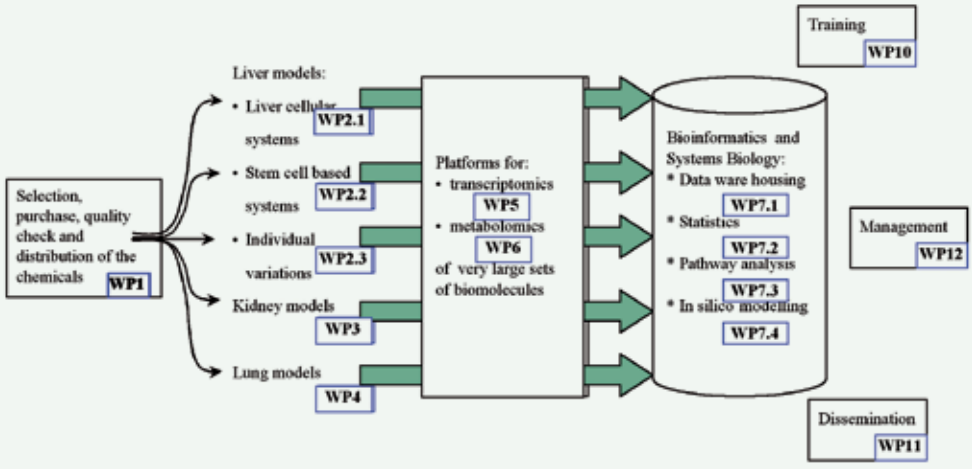
Figure 1. Overview of the workflow between the carcinoGENOMICS consortium.

WP1 Selection of chemicals

The main task of WP1 is therefore to select a set of model compounds, relevant to chemical carcinogenesis, according to pre-defined and well-rationalised selection criteria. WP1 also foresees purchase, distribution and purity testing of the selected chemicals.

Experimental design

Within the carcinoGENOMICS consortium, it was decided to envisage three classes of chemicals, namely (*i*) genotoxic carcinogens, (*ii*) non-genotoxic carcinogens, and (*iii*) non-carcinogens, thereby considering either ingestion (liver and kidney) or inhalation (lung) as principal route of exposure. Subsequently, selection criteria were defined, including (*i*) toxicological profile and gene expression profile, (*ii*) diversity and selectivity, (*iii*) biochemical and biophysical properties, and (*iv*) legal aspects and safety measures. For the purpose of finding chemicals that met the established selection criteria, public electronic databases were mined, such as the Carcinogenic Potency Database, the Carcinogenicity and Genotoxicity Experience Database, the National Toxicology Program database and the Chemical



Carcinogenesis Research Information System database. In addition, documents provided by regulatory bodies, including the International Agency for Research on Cancer and the Organization for Economic Co-operation and Development, were also consulted. Based on the information available, a preliminary set of common non-carcinogens, non-genotoxic carcinogens and genotoxic carcinogens was appointed. In-depth identification of the pre-selected compounds was then performed using the commercial (Chemical Abstracts Service) factual database SciFinder. Scientific literature was screened in parallel, thereby using PubMed as a bibliographical resource. All information obtained was then compiled into datasheets presenting compound-specific properties relevant to the selection criteria.

Results

The final list of compounds for the first phase of carcinoGENOMICS, as well as the selection criteria and strategy, has been published by Vinken et al. [1]. The list of chemicals is also presented in Table 1.

Table 1. List of compounds for the first phase of carcinoGENOMICS.

Compound	Organ involved
Genotoxic carcinogens	
Aflatoxin B1	Liver
4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone	Liver
2-Nitrofluorene	Liver, kidney
Benzo[a]pyrene	Liver, kidney, lung
Potassium bromate	Kidney
Streptozotocin	Kidney
1,3-Butadiene	Lung
Vinyl chloride	Lung
Sodium dichromate	Lung
Isobutyl nitrite	Lung

1 Vinken M., Doktorova T., Ellinger-Ziegelbauer H., Ahr H.J., Lock E., Carmichael P., Roggen E., van Delft J., Kleinjans J., Castell J., Bort R., Donato T., Ryan M., Corvi R., Keun H., Ebbels T., Atersuch T., Sansone S.-A., Rocca-Serra P., Stierum R., Jennings P., Pfaller W., Gmuender H., Vanhaecke T., Rogiers V. (2008) 'The carcinoGENOMICS project: critical selection of model compounds for the development of omics-based *in vitro* carcinogenicity screening assays' *Mutation Research Reviews* 629:202-210.

Compound	Organ involved
Non-genotoxic carcinogens	
Wy-14,643	Liver
Methapyrilene.HCl	Liver
Piperonyl butoxide	Liver
Sodium Phenobarbital	Liver
Tetradecanoyl phorbol acetate	Liver
Ochratoxin A	Kidney
Monuron	Kidney
Chlorothalonil	Kidney
Bromodichloromethane	Kidney
S-(1,2-dichlorovinyl)-L-cysteine	Kidney
2,3,7,8-tetrachlorodibenzo-para-dioxin	Liver, kidney, lung
Cadmium chloride	Lung
Sodium arsenate	Lung
Asbestos	Lung
Chloroprene	Lung
Non-carcinogens	
Nifedipine	Liver, kidney
Tolbutamide	Liver, kidney
Clonidine	Liver, kidney
Sodium diclofenac	Liver, kidney
D-Mannitol	Liver, kidney
Ethylene	Lung
Beclomethasone diproprionate	Lung
Ipratropium bromide monohydrate	Lung

Next steps

An additional set of compounds will be used for the fine-tuning of the *in vitro* tests as developed by WPs 2-4. This second list of chemicals is currently being composed, whereby an identical selection strategy as described above is followed.

WP2 Liver models

The liver is a primary site for drug metabolism and is frequently involved in adverse chemical reactions. Consequently, research questions on toxicogenomics of the liver are essential in the development of any predictive *in vitro* model. Our objective is to develop an *in vitro* hepatic system showing the appropriate features of the organ *in vivo* to be considered a substitute of animals in toxicological studies of chemical testing. Different *in vitro* liver models are considered: HepG2+/- transcription factors, HepaRG, rat hepatocytes +/- HDAC inhibitor, as well as human embryonic stem cells derived hepatocyte like cells.

Experimental design

Table 2 shows the methods used within carcinoGENOMICS' WP2.

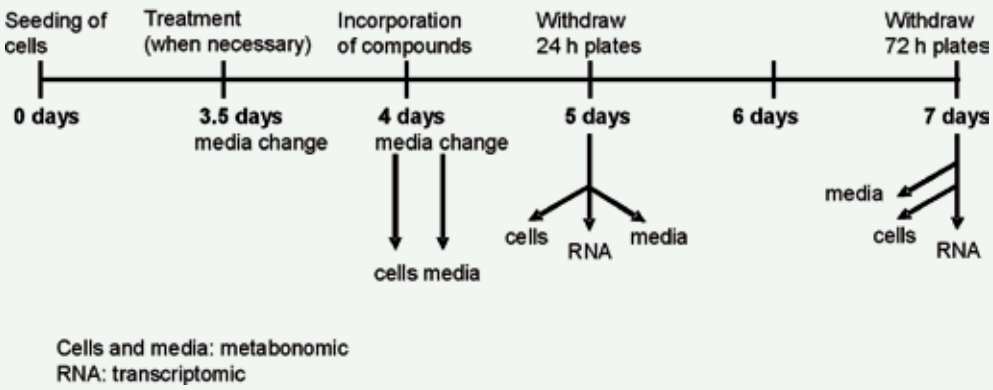


Figure 2. WP2-Study design: general overview.

Table 2

Name of the test method	–
Clinical endpoint	Carcinogenesis.
Cell (line)	Rat hepatocytes, HepG2 (human derived), HepaRG (human derived) and hepatocyte-like cells (derived from hESC); RPTEC/TERT1 cells; primary human bronchial epithelial cells.
Method description	Incubation of selected carcinogens with the different cell models.
SOP	No.
Endpoints	Transcriptomic and metabonomic analysis.
How is a positive result defined?	PCA should classify the carcinogens into their specific group (genotoxic or non-genotoxic). Transcriptomic and metabonomic data will reveal markers (genes and compounds) usable in the screening of chemicals.
How is a positive result expressed?	New carcinogenic markers to develop a 'carcinochip' for evaluation of chemicals.
Applicability	Carcinogenic test of chemical compounds (fertilizers, drugs, etc.).
Positive control	Selected and known carcinogens, e.g. genotoxic carcinogens: NNK, 2-nitrofluorene, Benzo[a] pyrene; non-genotoxic carcinogens: Methapyrilene. HCl, Sodium phenobarbital, Piperonylbutoxide.
Negative control	Non-carcinogens: Nifedipine, D-mannitol, Sodium diclofenac.
Performance	Sensitivity, specificity, accuracy, positive and negative predictive value.
Can the test method be used in a regulatory safety context?	Yes. ECHA/EFSA/EMEA/OECD guidelines for assessing genotoxicity and carcinogenicity.
Which R would the test method impact?	Replacement.
How can the test be used?	The test can be used as a stand-alone or can be added to the currently existing *in vitro* test battery for assessing genotoxicity and potential carcinogenicity.
Other important remarks?	Cellular models will be organ-specific, addressing the major target organs for chemical carcinogenesis *in vivo*, e.g. the liver, the kidney and the lung.

Results

Incubation conditions for the different *in vitro* liver models have been optimised. In order to select the best model, assays have been challenged by Phase I model compounds (see WP1) and are now awaiting genomics analyses. The following is an update of the current analysis of the six pre-selected models incubated with positive and negative controls (see Table 3).

Next steps

- Finish the preparation of the metabonomic data;
- Select the best liver model(s);
- Repeat the experiments with an extended number of compounds.

Table 3

Model	Treatment	Transcriptomic	Metabonomic
Primary hepatocytes		132 (55%)	80 (33%)
Primary hepatocytes	+HDAC inh.		
HepG2		240 (100%)	0 (0%)
HepG2 (upgraded)	+ LETF		
HepaRG		120 (100%)	120 (100%)
hESC derived HCLC		120 (100%)	0 (0%)

WP3 Kidney models

Renal cancers mainly arise in the proximal tubule. There is very little knowledge on causative agents for renal carcinogens in humans but animal studies have identified a number of renal carcinogens. The main objective in the renal models component of this project is to establish robust renal cell culture models to assess the effects of selected model carcinogens and develop predictive mechanistic-based *in vitro* tests that are representative of various modes of carcinogenic action.

Experimental design

Renal epithelial cells of human and rat origin are being used to allow inter-species comparison between the selected carcinogens. Both human and rat cell models have been developed. Primary human proximal tubular cells were prepared from kidneys obtained from patients undergoing nephrectomy. These cells were compared to the well established human proximal tubular cell line HK-2 but also to newer cell line RPTEC/TERT1.

Results

The RPTEC/TERT1 cells retained many of the characteristics of the *in vivo* renal proximal tubule. These cells were capable of transporting fluid and electrolytes across the epithelial layer and showed a very good transepithelial electrical resistance (TEER). These cells also showed excellent staining for epithelial markers such as E-cadherin and occludin. The RPTEC/TERT1 cells have distinct advantages for this carcinogenomics project in that they have not been transformed using an oncogene. The RPTEC/TERT1 cells become more 'differentiated' after 10 days in culture and consume no glucose and produce no lactate. The rat cell line NRK-52E was also shown to exhibit good epithelial characteristics and generated a TEER. The conditions have been optimised and standardised for human and rat cell lines and SOPs have been developed. Karyotype analysis of the RPTEC/TERT1 and NRK-52E cells demonstrated that these cell lines had the appropriate median number of chromosomes. However, the HK-2 human cell line had a very abnormal chromosome complement. Therefore it was decided not to continue with the HK-2 cell line but to go forward with the RPTEC/TERT1 cells. A series of concentration and time dependent experiments were carried out with the selected compounds identified in WP1. It was agreed that we would determine the IC50 and IC10 (defined as the concentration to reduce cell viability to 50% and 10% respectively). A series of experiments were also carried out to investigate the xenobiotic metabolising enzyme capability in the cell models. No significant enzyme activity was detected. Alternative strategies are being developed to overcome the lack of xenobiotic metabolising enzymes. These include i) coculture of human kidney cells with Hepa RG cells, ii) use of human S9 fractions with human kidney cells, and iii) coculture of NRK-52E cells with CHO cells transfected with CYPs. Experiments were carried out to detect possible lists of indices of carcinogenicity based on cytomic assays. The results to date with the 'differentiated' RPTEC/TERT1 cells indicate a switch to lactate production may be a very useful indicator. Increased expression of vimentin and decreased expression of E-cadherin in RPTEC/TERT1 cells also seem promising as indicators of carcinogenicity. We have found that the NRK-52E cells are very suitable for the automated *in vitro* micronucleus assay and it is anticipated that this assay will be useful for detecting carcinogenicity by compounds.

Next steps

Genomic and metabonomic experiments will be carried out using the selected carcinogenic compounds. Results from these will be compared with cytomic assays for carcinogenicity and predictive models will be developed arising from the results.

WP4 Lung models

The overall objective of WP4 is to develop a detailed understanding of the pivotal role played by lung epithelial cells (EC) in the induction of lung carcinogenesis. This will lead to an *in vivo*-like cell-based tests system for assessing the carcinogenic (genotoxic and non-genotoxic) potency of chemicals when exposed to the respiratory system.

Experimental design

1. Differences between primary human bronchial EC, and various bronchial and alveolar cancer cells (as provided by available cell lines) are described by genomics in order to map the various proteins, pathways and networks that are affected during carcinogenesis;

2. A cell-based assay is developed using primary human bronchial cells. The minimal requirements for the assay include: 1. Cells not exceeding four passages; 2. Proper barrier-formation, evidenced by the **T**rans-**E**pithelial **E**lectric **R**esistance (TEER); 3. Measurable metabolic activity;

3. The cell culture conditions reported by Epithelix (http://www.epithelix.com) to maintain viable primary human bronchial cell cultures for up to one year are evaluated in order to provide cell culture conditions allowing long-term and/or repeated exposure to chemicals;

4. The primary cells are subjected to Hu tTert immortalisation in order to obtain an immortalised non-carcinogenic cell line;

5. Potential markers for early events in carcinogenesis identified by differential analysis of the genomic data are evaluated for their *in vivo* relevance using the precision cut lung slices (PCLS) technology;

6. The predictive accuracy of selected markers for early events in carcinogenesis is evaluated on an extended panel of chemicals;

7. Eventually, the selected test system is refined and optimised to meet with the overall objectives of the project.

Results

The genomic profiles for human primary cells and the human cancer cell lines A549 and Calu-3 are available, and differences are identified. A cell-based assay using primary human bronchial cells with the characteristics described in *Experimental design* (b) is now available. Currently, this assay is subjected to the learning panel of chemicals provided by WP1. The cell culture conditions provided by Epithelix are currently evaluated in terms of their impact on the responsiveness to known chemicals (carcinogens, sensitisers and irritants). The immortalisation of the primary human bronchial cells used in (b) using the Hu tTert approach is ongoing.

Next steps

The chemicals proposed by WP1 are assessed in the cell-based assay (b) (as soon as available (d)) and genomic analysis is performed (WP5). Potential markers for early events in carcinogenesis identified by differential analysis of the genomic data are evaluated for their *in vivo* relevance using the precision cut lung slices (PCLS) technology. The predictive accuracy of selected markers for early events in carcinogenesis is evaluated on an extended panel of chemicals. Eventually, the selected test system is refined and optimised to meet with the overall objectives of the project.

WP5 Transcriptomics

The major goal of carcinoGENOMICS is to develop a battery of mechanism-based *in vitro* tests based on the application of 'omics' technologies (i.e. genome-wide transcriptomics as well as metabonomics) to robust *in vitro* systems (rat/human). The main task of WP5 is the generation of the transcriptomics profiles from samples generated by WPs 2-4, and to make these data available to WP7 for further bioinformatic analyses.

Experimental design

The partners from WPs 2, 3 and 4, where all the biological experiments will be conducted, were provided with standardised protocols plus training for RNA isolation, storage and shipment to the Maastricht University. First, rigid quality control of the RNA samples is conducted by using the Agilent BioAnalyzer and UV spectroscopy, in order to provide information on purity, integrity and concentration of the RNA samples. Thereafter, microarray experiments are conducted, which consist of labelling RNA samples with fluorophores (using Affymetrix kits), hybridisation of labelled samples on the Affymetrix GeneChip arrays, scanning the arrays, image analyses/feature extraction to quantify the expression for each gene. Finally, the raw transcriptomic data are uploaded onto the project's data warehouse at EBI.

Results

Since April 2008 RNA samples have been sent to Maastricht for gene expression profiling; 429 samples were received until March 2009. The quality of these RNA samples is in general very good. The ratio Absorbance 260nm / Absorbance 280nm is well above the minimum value of 1.8, and the RIN (RNA Integrity Number) provided by the Agilent BioAnalyzer are almost all above the minimum value of 8.0. From these, 247 have been analysed by DNA microarrays by March 2009. No major technical problems were encountered, except that one sample did not yield enough cRNA during the procedure (the sample with the lowest RIN, it has been hybridized anyway). The quality control data generated by the Affymetrix scanner, based on scaling factor, background signal, present calls for probe sets and 3'-5' expression ratios for control genes, indicate that all data sets are of good quality, except for the sample with insufficient cRNA.

The generated gene expression profiles (the so-called cel-files), together with all descriptive data about cellular experiments (as received from the partners of WP2-4) and about the array experiments (as generated in WP5), are uploaded to the ftp-site at EMBL-EBI. In that way they are handed over to WP7 for further analyses (quality control, data analysis and data mining).

Next steps

As the cell systems are exposed to the selected compounds, more samples will be generated by WPs 2-4 for transcriptomic analysis. In total, 2160 microarrays were conducted in the first years of this project.

WP6 Metabonomics

Within the carcinoGENOMICS project, the generation of metabonomic profiles is an important addition to transcriptional profiling, giving the project phenotypic information on the effects of potential carcinogens on the metabolism of cells. The main task of WP6 is the generation of these profiles from samples generated by WPs 2-4.

Experimental design

Once samples generated by WPs 2-4 have been shipped to WP6 they are prepared for metabonomic analysis by 1H Nuclear Magnetic Resonance (NMR) Spectroscopy. Where possible, both intracellular extracts and culture media are studied across multiple timepoints. Once NMR spectra have been obtained for each of the samples, pattern recognition and other statistical approaches are applied to extract differences between the treatments across the data and to deconvolve these differences from other factors such as time and growth rate. All data is uploaded to the consortium database managed and further analysed by WP7.

Results

Metabonomics has proven to be useful in evaluating the different cell systems and procedures within WPs 2-4. As an example, one study explored the effects of a histone deacetlyase inhibitor, TSA (Tricostatin A) in cultured primary hepatocytes, which stabilises the epigenome and xenobiotic metabolic capacity but has an unknown effect on endogenous metabolism. Metabolic differences over time with TSA exposure were less than those observed in control, suggesting that TSA stabilised the metabolome of primary hepatocytes *in vitro*. Our measurements indicated that the bioavailability of GSH is higher in TSA treated cells: this would have implications in many metabolic processes and specifically phase II conjugation of xenobiotics. Levels of choline metabolites, frequently altered as cells are transformed to an immortalised or tumourigenic phenotype, were also affected. Overall our data suggest that TSA treatment reduces the loss of a normal metabolic phenotype in cultured primary hepatocytes, improving the model as a tool to study endogenous liver metabolism and potentially affecting the accuracy of all biological assays in this system.

A second study examined the different metabolic phenotypes seen in two competing models for *in vitro* assessment of carcinogenicity in the kidney, HK2 and a newly developed line, RPTEC/TERT1. The RPTEC/TERT1 line is immortalised only by the ectopic expression of telemorase in contrast to HK2 which is immortalised through transfection with E6 and E7 from the human papilloma virus 16, which causes inactivation of the tumour suppressors P53 & Rb. Metabonomic analysis showed that glycolysis and branched chain amino acid degradation were major pathways differentiating the metabolism of these models.

Next steps

As the cell systems are exposed to the selected compounds more samples will be generated by WPs 2-4 for metabonomic analysis. In addition, as parallel sets of data from transcriptomics and metabonomics become available within the consortium, work on data integration methods for combining this knowledge is also underway.

WP7 Bioinformatics and Systems Biology

The main objective of the bioinformatics and computational modelling groups is the integration of the heterogeneous project data into response models on different levels of granularity in order to predict the carcinogenic effect of chemical compounds. Through extensive analysis of molecular expression data, the groups will identify marker genes, pathways and functional modules that are predictive for carcinogenicity.

Experimental design

Major elements of the carcinoGENOMICS bioinformatics approach are proper data integration of the various experimental data, the bioinformatics analysis, the annotation and analysis of molecular pathways and the subsequent computational modelling of pathways and pathway modules.

Results

The European Bioinformatics Institute (EBI) has developed a project infrastructure providing common structured representation and storage mechanisms for a range of diverse omics-based data associated with phenotypical information. This infrastructure provides a means for the scientific community and the regulatory authorities to access high quality data in a well structured standard format to assess the validity of the developed prediction models. It is based on the BioInvestigation Index (BII), a standard-compliant prototype infrastructure and includes a standalone editor tool that enables researchers to *structure* and *edit* the experimental metadata in a common format (ISAcreator), and a repository providing access to the information via a web-based user interface and web services. A prototype instance of the BII infrastructure is available (http://www.ebi.ac.uk/bioinvindex).

The Max Planck Institute for Molecular Genetics (MPIMG) has performed a survey of pathway databases and modelling tools that are useful for the mechanistic analysis of carcinogenesis. These pathway databases have been integrated into a unified schema and the integrated content can be publicly accessed via the ConsensusPathDB (http://cpdb.molgen.mpg.de). ConsensusPathDB has been used within the carcinoGENOMICS project for the identification of functional modules that are affected by the different chemical treatments. Such topological analysis is the linking step between high throughput experiments and computational modelling. Functional modules have been annotated to a response model connecting various endpoints of carcinogenesis within the PyBioS modelling system (http://pybios.molgen.mpg.de).

Next steps

Further integration and combined analyses of molecular expression data will be used to identify classifier systems on a statistical and mechanistic level.

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PREDICT-IV

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

Grant Agreement 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/

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 methods, a better prediction of toxicity of a drug candidate will be developed. By combining analytical chemistry, cell biology, and mechanistic toxicology and *in silico* modeling with new advanced technologies, such as 'omics' and high-content imaging, a link between classical *in vitro* toxicology and modern systems biology will be set. The integration of systems biology into predictive toxicology will lead to an extension of current knowledge in risk assessment. As a result, new tailored non-animal testing strategies will be derived. This will enable pharmaceutical companies to take the decision on exclusion of drug candidates due to unacceptable toxicity well in advance of performing animal safety testing. PREDICT-IV evaluates the toxicity of the most frequently affected target organs such as kidney and liver, additionally complemented by neurotoxicity assessment using newly developed *in vitro* neuronal models.

The ultimate goal is to provide an integrated testing strategy with defined specific safety markers to predict toxicity prior to pre-clinical animal testing. This may result in a reduction of animal experimentation by using an *in vitro* approach and other alternative methods such as *in silico* modeling for a substantial improvement towards the 3Rs principles.

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

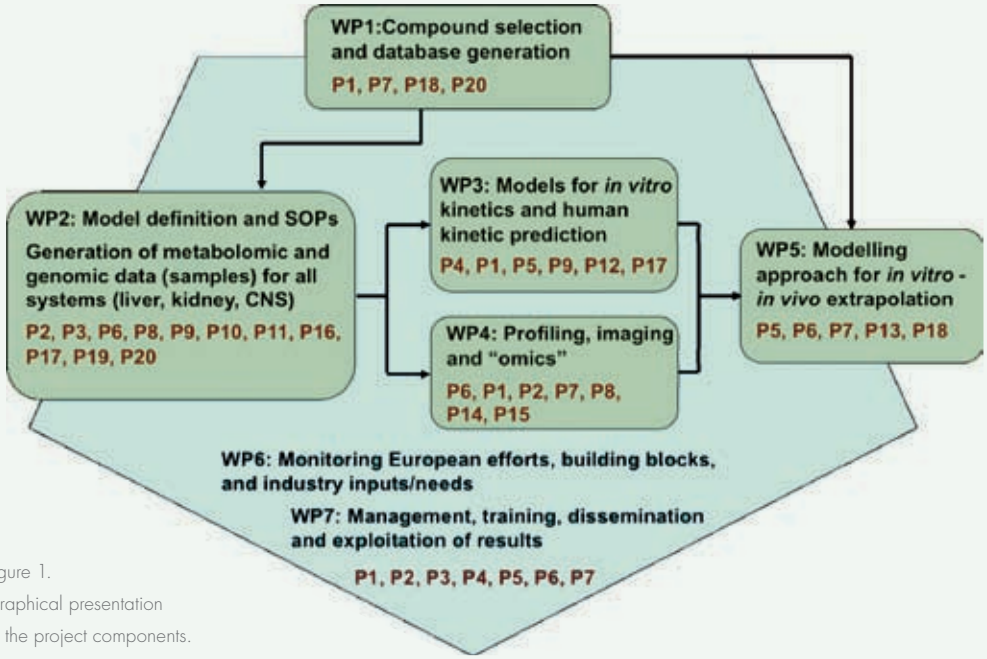

Figure 1.
Graphical presentation
of the project components.

The scientific objectives for the project are the following:

- 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 only lead 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 was further aligned to integrate different technologies used within the project as well to facilitate a standardised approach for the several 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 is acquired and utilised by project partners.

The toxicity testing strategy is based on a systems biology approach. The end-points 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 medium proteins, and in addition may be unstable or metabolised, the actual concentration of a compound in cell culture medium is not necessarily the amount applied. Thus, we will measure the actual concentration of parent compound and their 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 based on the following 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*);
- ideally 'pairs' of compounds (toxic – 'non-toxic' and parent – metabolite) if available;
- biochemical and biophysical properties.

Cell culture models

In the first period of the PREDICT-IV project, a major focus was the optimisation, standardisation and characterisation of the long-term human models for hepatotoxicity, human based cellular systems for nephrotoxicity testing and *in vitro* models for CNS toxicity testing.

Long-term human models for hepatotoxicity

In a first step, the standard operating procedures (SOPs) for culture of long-term human and rat liver cell systems were developed. Two plate formats were compared, 24-well and 96-well plates (higher throughput plate format), in order to assess the cytotoxicity of the test compounds. Different configurations with collagen and/or matrigel were tested. A so-called 'Oil slick' method, whereby low concentrations of matrix are diluted into the culture medium (rather than establishing a gel), was also tested at different concentrations with both collagen and matrigel.

In the next steps, the characterisation of human and rat hepatocytes will be done for specific activities of the major CYPs involved in the metabolism of xenobiotics (CYP1A2, CYP3A, CYP2B and CYP2C). Optimisation of the down-scaling of the plate format (from 24- to 96-well) and the use of substrate cassette incubations of four substrates combined with LC/MS analysis allows for the determination of all four CYPs activities in one well and a reduction in cell usage. The kinetics of the metabolism of each substrate (15, 30, 60 and 90 minutes) is being determined using substrates incubated alone or in a cocktail mixture. Five different cocktails of the same substrates (phenacetin, bupropion, midazolam and diclofenac) have been tested. The cocktail concentrations were based on Km and Vmax, values and equal concentrations of substrates. The effect of the phase II enzyme inhibitor, salicylamide (0.5, 1, 2, 3mM), has also been determined.

Human based cellular systems for nephrotoxicity testing

The main cell model has been selected as the RPTEC/TERT1 cells. These cells form monolayers, become contact inhibited and differentiate into a stable low glycolytic phenotype. Additionally, they exhibit many characteristics of the proximal renal tubule *in vivo*. RPTEC/TERT1 can be maintained indefinitely as a tissue monolayer, thus allowing long-term exposures to toxins.

SOPs have been drawn up for cell culture medium preparation (serum free hormonally defined), cell culture and also differentiation. Further characterisation of the cells has been performed, showing that differentiation results in an alteration 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, protein biosynthesis and antioxidation and free radical removal.

The testing of selected compounds has begun in 96 well-based, dose range finding experiments.

The next steps will be to establish definitive exposure protocols for all compounds to be brought to metabolomic and transcriptomic analysis.

Definition of standard strategies to support in vitro *kinetics*

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

The chemical stability depends strongly on the experimental conditions (i.e. pH, temperature, light, duration and pO_2) used during the test. A significant change of the test chemical concentration has to be taken into account and to be corrected during subsequent calculations.

- Adsorption to physical components

Adsorption of the test chemicals to plastic flasks or plastic dishes reduces the free concentration and makes a corrective measurement or an estimation of the loss necessary.

- Binding to medium macromolecules, essential proteins

A possible binding of the compounds to medium components has to be taken into account. In addition, the possible non-linearity due to saturation of binding sites requires a measurement of the amount bound for the concentration range used within the experiments.

- Free vs. bound concentration over time

Since the unbound concentrations are considered as the relevant variable for the exposure of *in vitro* systems, the measurement of both peak and average concentrations over time (similarly to AUC *in vivo*) are recommended.

- Interaction with cell components

An accumulation of the test chemical within the cells or cell walls needs to be measured via selected testing methods to describe the kinetics of the parent compound.

- Metabolic competence

The kinetic of metabolites over time is considered in a long-term experiment as very important and implies a necessary cellular

metabolic competence characterisation. Serial sampling of medium from the *in vitro* systems will be performed and the concentrations of parent drug and/or metabolites in medium have to be measured by selected detection methods.

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

  To ensure that the transport of chemicals is guaranteed through an active process by transporters, the presence of the main families of transporters has to be known. This ensures that the applied drugs will pass the cell membrane not only by passive diffusion. The expression of the main families of transporters can be checked by molecular biology techniques (RT-PCR) or immune biology (Western Blot) techniques.

- Metabolic competence

  There is also a need for the characterisation of the presence of drug metabolising enzymes. Both phase I and phase II enzymes have to be considered and, in addition to their presence, their function has to be controlled by known substrates that can be detected.

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

- Transport mechanism across cell membranes of the test chemical

  Serial sampling of the medium for selected concentrations with subsequent measurement of their disappearance may reveal the mechanism through which the chemical passes across the cell membrane. An alternative approach is measuring the presence of the parent compound within the cell. This information will allow distinguishing between an active and a passive transport into the cell.

- Biotransformation pathways

The concentration of the substrates and their metabolites can be obtained with serial sampling of medium from the *in vitro* systems in which the concentration of parent drug and/or metabolites will be measured. This requires selected detection methods and a serial sampling of the biological components of the system. Information on the natures of the metabolites, the concentration to be tested, and differences between organs can be preliminary acquired by using subcellular fractions.

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2.4 Computational modelling and estimation techniques

The project presented here, OpenTox, aims to facilitate the access to unified toxicological data and quantitative structure-activity relationship ((Q)SAR) models, thus promoting the development, acceptance and implementation of (Q)SAR models and algorithms.

OpenTox

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

Grant Agreement Number: HEALTH-F5-2008-200787
Project Type: Collaborative Project (FP7)
EC contribution: € 2 975 360
Starting date: 1 September 2008
Duration: 36 Months
Website: http://www.opentox.org

Objectives

The goal of the OpenTox project is to develop a predictive toxicology framework that provides a unified access to toxicological data, (Q)SAR models and toxicological information.

The OpenTox framework will provide tools for the integration of data from various sources (public and confidential), for the generation and validation of (Q)SAR models for toxic effects, libraries for the development and seamless integration of new (Q)SAR algorithms, and scientifically sound validation routines. OpenTox will attract users from a variety of research areas:

- toxicological and chemical experts (e.g. risk assessors, drug designers, researchers);
- (Q)SAR model developers and algorithm developers;
- non-(Q)SAR specialists requiring access to Predictive Toxicology models and data.

The OpenTox project will move beyond existing attempts to solve individual research issues within this area, by providing a flexible, extensible, and user-friendly framework that integrates existing solutions as well as providing easy access to new developments.

OpenTox will be relevant for the implementation of REACH as it allows regulatory and industrial risk assessors to access experimental data, (Q)SAR models and toxicological information from a unified, simple-to-use interface, that adheres to European and international regulatory requirements (e.g. Organisation for Economic Co-operation and Development (OECD) Guidelines for (Q)SAR validation, and QSAR Model Reporting Formats (QMRF)). For maximum transparency, OpenTox will be published as an open-source

project. This will allow critical evaluation of the implemented algorithms, ensure widespread dissemination and attract external developers. Facilities for the inclusion of confidential in-house data and for accessing and integrating commercial prediction systems will be included.

The OpenTox framework will be populated initially with high-quality data and (Q)SAR models for chronic, genotoxic and carcinogenic effects. These are the endpoints, where computational methods promise the greatest potential reduction in animal testing that would be required for the implementation of REACH. The impact of OpenTox, however, will go beyond REACH, industrial chemicals and long-term effects, because reliable toxicity estimates are also needed for other products (e.g., pharmaceuticals, cosmetics, and food-additives) and endpoints (e.g., sensitisation, liver-toxicity, and cardiotoxicity).

The proposed framework will actively support the development of new (Q)SAR models by automating routine tasks, providing a testing and validation environment and allowing the easy addition of new data. It will also support the development of new algorithms and avoid duplicated work by providing access to common components, validation routines and an easy comparison with benchmark techniques. For this reason, we expect that OpenTox will lead to (Q)SAR models for further toxic endpoints and generally improve the acceptance and reliability of (Q)SAR models.

Experimental Design

Initial research has defined the essential components of the OpenTox framework architecture, the approach to data access, schema and management, the use of controlled vocabularies and ontologies, Web service and communications protocols, and the selection and integration of algorithms for predictive modelling.

OpenTox is placing a strong emphasis on close interaction with users so that development tasks and releases are guided and prioritised by user requirements and challenges in safety analysis and risk assessment. A user perspectives workshop was held in Basel, Switzerland right at the start of the project in September 2008 with participation from the European Chemicals Bureau, Nestlé, Lhasa, the OECD, CADASTER and Bayer Healthcare. Analyses of use cases are in progress and include cases for REACH-relevant risk assessment, chemical categorisation and prioritisation, drug development, and food safety evaluation, with the resulting requirements guiding framework design and initial application development.

Use cases are also being prioritised so as to satisfy REACH requirements. A two-day workshop on REACH Requirements in Predictive Toxicology will be held at the Istituto Superiore di Sanità in Rome on 10 and 11 September 2009. Participants at the workshop will involve cross-industry users and regulatory experts, in addition to developers who will discuss the requirements and use cases of users in chemical toxicology evaluation and risk assessment. The workshop format will structure the activity around sets of short presentations providing user, regulatory and solution perspectives, followed by knowledge cafe discussions of challenges, issues, solutions, and ways forward in small groups. Interested parties should contact the Project Coordinator for potential participation.

OpenTox leadership places a strong emphasis on the value of collaboration approaches and is constantly reaching out, contacting and meeting with potential partners including other EC projects, Open Source initiatives, software development vendors, and experimental toxicologists. During the initial months of the project, OpenTox involved representation from OSIRIS in its workshop activities, met with the partners of the FP6-funded Chemomentum project, held meetings or virtual conferences with most of the relevant leading

software vendors in QSAR-based toxicology, and met with leading US principal academic investigators and representatives from the US FDA, EPA and NIH.

OpenTox is collaborating closely with the FP7-funded CADASTER project (http://www.cadaster.eu) which is a 'sister project' funded under the Environment theme. CADASTER will provide practical guidance to integrated risk assessment by carrying out a full hazard and risk assessment for industrial chemicals. The project will develop a Decision Support System that will be updated on a regular basis in order to accommodate and integrate emerging practices and procedures for alternative non-animal based testing methods. OpenTox and CADASTER partners are working closely so as to promote and develop common practices, standards and procedures in the area of *in silico*-based predictive toxicology approaches responding to user requirements in the area of REACH-relevant risk assessment. The collaboration should enable the development of a leading platform supporting the safety evaluation and regulatory compliance needs of European industry.

OpenTox partners are progressing QSAR model development through international collaboration and participation in evaluating and testing models against toxicological data produced from the EPA's ToxCast program. Such models offer the promise of developing the capability of predicting *in vivo* toxicology endpoints based on a combination of *in vitro* data and *in silico* modelling, which would enable the goal of reduced animal testing in addition to improving understanding on the mechanism of action.

OpenTox is pursuing an open community-based approach to technical development. External developers may participate in technical discussions through the Web forums, mailing list and virtual meeting facilities. The growing OpenTox technical documentation is also made transparently available through the website. OpenTox components and services may be combined with external provider resources for the creation of new services.

Results

The OpenTox repository and website (http://www.opentox.org) was established in autumn 2008 and is being used for partner and community communications, in addition to documentation of all collaborative technical developments of the project. Descriptions of current standards and existing software components that are relevant for OpenTox applications have been made available on the OpenTox website.

Based on initial design work, it was agreed that OpenTox will be a platform-independent collection of components that interact via well-defined interfaces. The preferred form of communication between components will be through Web services. A set of minimum required functionalities for OpenTox components of various categories (prediction, descriptor calculation, data access, validation, and report generation) has been published on the website.

An analysis of several existing toxicological databases relevant to REACH has been carried out, which has determined a suitable approach to OpenTox ontologies, data schema and reporting templates, satisfying both effective scientific data management and international guidelines.

We have completed a detailed and comprehensive documentation of the algorithms and implementations relevant for initial OpenTox applications, accompanied by the development of standard templates and selection criteria for algorithms.

An inventory of software components that initially includes a significant collection of software from OpenTox partners, which is being incorporated into the platform, has been documented on the OpenTox website and includes the following components.

Prediction

Table 1. List of Current Prediction Components.

Name	Component Description
Rumble	RUMBLE (RUle and Margin Based LEarner) is a statistically motivated rule learning system based on the Margin Minus Variance (MMV) approach. It is set up very flexibly as it can make use of different plug-ins (e.g. FTM plug-in, PROLOG plug-in, Meta plug-in) for different kinds of rules.
Toxmatch	Provides means to compare a chemical or set of chemicals to a toxicity dataset through the use of similarity indices.
Toxtree	Toxtree is a full-featured and flexible user-friendly open-source application, which is able to estimate toxic hazard by applying a decision tree approach. Currently it includes five plug-ins.
iSar	Perl implementation of a lazy SAR algorithm.
Lazar	Lazar command line program; C++ implementation of various lazar algorithms.
Lazar Web interface	Web interface for lazar.

Descriptor Calculation

Table 2. List of Current Descriptor Calculation Components.

Name	Component Description
AMBIT	A relational database schema, allowing users to store and query all relevant structure and property information, including data for toxicity endpoints from various sources and formats. Can handle a very large number of structures efficiently. Functional modules allowing a variety of evaluations, flexible structure, similarity and other information retrieval. Used in both stand-alone and Web (servlets/taglibs-based) applications.
FreeTreeMiner	The FreeTreeMiner (FTM) software computes all subtrees (substructures) occurring at a given minimum frequency in a set of molecules. The subtrees are built via a depth first search (DFS). In addition to the minimum frequency support, a maximum frequency constraint can be set.
LibFminer	LibFMiner implements a method for efficiently mining relevant tree-shaped molecular fragments, each representing a geometrical class, with minimum frequency and statistical constraints.

Name	Component Description
Toxtree	Toxtree is a full-featured and flexible user-friendly open source application, which is able to estimate toxic hazard by applying a decision tree approach.
gSpan'	C implementation of a graph-mining algorithm-feature generation: mining for frequent subgraphs or subpaths/subtrees.
Lazar	Lazar command line program; C++ implementation of various lazar algorithms: feature generation (paths); nearest neighbour and kernel classification and regression; local models; activity-specific similarities.
MakeMNA	MakeMNA is a software product for generating MNA descriptors. These descriptors are based on the molecular structure representation, which includes the hydrogens according to the valences and partial charges of other atoms and does not specify the types of bonds.
MakeQNA	Quantitative Neighbourhoods of Atoms (QNA) descriptors are based on quantities of ionisation potential (IP) and electron affinity (EA) of each atom of the molecule.
MakeSCR	Delphi implementation of a self-consistent regression (SCR) algorithm.

Data Access

Table 3. List of Current Data Access Components.

Name	Component Description
AMBIT	Relational database schema, allowing user to store and query all relevant structure and property information, including data for toxicity endpoints from various sources and formats. Can handle very large number of structures efficiently.
DSSTox data for lazar	Git repositories for versioned DSSTox SDF files, conversion scripts to generate lazar input files, validation results.
Sens-it-iv internal database	Internal database for the Sens-it-iv (http://www.sens-it-iv.eu) FP6 project.
Toxmatch	Provides means to compare a chemical or set of chemicals to a toxicity dataset through the use of similarity indices. Intended use is one-to-many or many-to-many quantitative read-across. To help in the systematic formation of groups and read-across.

Report Generation

Table 4. List of Current Report Generation Components.

Name	Component Description
AMBIT	recording of user actions; improved data entrance and visualisation; reporting compatible with IUCLID 5.
Lazar Web interface	Report Generation.

Validation

Table 5. List of Current Validation Components.

Name	Component Description
Lazar	**Leave-one-out validation.** *Input:* chemical structures and activities. *Output:* actual values versus vpredicted values, validation statistics.

Integration

Table 6. List of Current Integration Components.

Name	Component Description
OpenTox plug-in	Ruby on Rails plug-in with interfaces to R, OpenBabel, CDK and basic functionality to create predictive toxicology applications.
Lazar plug-in	Ruby on Rails plug-in with interfaces for the lazar command line program: Web-based GUI; rake tasks for administration and validation.
Lazar Web interface	Web interface for lazar.

The initial evaluation, design, and analysis work carried out in the initial six months of the project has been published in the following four initial OpenTox reports:

- 'Initial requirements, standards and APIs';
- 'Project repository and website';
- 'Initial ontologies for toxicity data';
- 'Report on algorithm evaluation and selection'.

Next Steps

The initial prototype implementation of the OpenTox Framework is scheduled for release for early 2010. The framework will support the development of *in silico* predictive toxicology applications based on OpenTox components for data management, algorithms and validation. Beta test applications will be made freely and openly available to users from mid-2009 through the OpenTox website and linked services including partner resources. Such early applications will support users in the development and training of QSAR models against their own toxicological datasets, e.g. they may upload a dataset for a given endpoint to the OpenTox website, select a variety of parameters and build and download a model. Subsequent releases in 2010 and 2011 will extend the use cases and applications supported, refine interface designs and report generation, address handling of confidential data, extend validation resources, and evaluate the effectiveness of the (Q)SAR methods against specific REACH-relevant endpoints.

The OpenTox Framework and initial applications will be presented by the coordinator Barry Hardy (Douglas Connect) as an invited keynote seminar at the Fifth International Symposium on Computational Methods in Toxicology and Pharmacology Integrating Internet Resources (CMTPI 2009) in Istanbul, Turkey in July 2009, at the European Society of Toxicology international congress in Dresden, Germany in September 2009, and at the InnovationWell community of practice meeting in Philadelphia, US in October 2009.

Advisory Board

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2.5 High throughput techniques

The three projects of this section have the common goal of developing high throughput techniques to use for toxicity assays.

TOXDROP, a two-year project concluded in 2006, developed an innovative 'Cell-on-Chip' technology to screen pesticides for toxicity, which is now being used in further studies for evaluating the effect of nanoparticles on human health.

COMICS aims to increase the throughput and speed of scoring of the comet assay for DNA damage.

NanoTEST is focused on developing high throughput testing strategies using *in vitro* and *in silico* methods to assess the toxicological profile of nanoparticles.

TOXDROP

Innovative 'Cell-on-Chip' technology to screen chemicals for toxicity, using cultured cells within tiny 'nanodrops' of culture fluid

Contract number: SP22-CT-2004-513698
Project type: Specific Targeted Research Project (FP6)
EC contribution: € 1 615 887.89
Starting date: 1 January 2005
Duration: 24 months
Website: http://toxdrop.vitamib.com

'Cell-on-Chip' technology follow-up

Implementing highly parallel cell-based assays that would replace animal testing is crucial for evaluating the effect of nanoparticles on human health. The Specific targeted research project (STREP) TOXDROP (2005-2006) has previously developed innovative 'Cell-on-Chip' technology to screen pesticides for toxicity, using cultured cells within tiny drops of culture fluid. We have been using the 'nanodrops' concept to establish in a massively parallel standardised manner the relationship between cell phenotype and **toxicity of nanoparticles** in the **project PARTOX** funded by the ANR in 2008. Contact Jesus. Angulo@ensmp.fr for more information.

We expect this new concept of cell-based toxicity screening in Cell-on-Chips to allow us to determine *in vitro* the biohazard of nanoparticles. The combined knowledge of the ANR-PARTOX consortium in bio-informatics, physicochemistry, toxicology, image processing and cell-based assays is ensuring that *in vitro* tests are set up to a high standard for evaluating the risk nanoparticles in bronchial cells. This rich environment is helping the PARTOX consortium to answer the particular question: **what risk assessment can we make for man-made nanoparticles by using a combination of nanoparticle description, high throughput screening and high content analysis?**

Preliminary results: Partners CEA and ARMINES have started to implement the innovative Cell-on-Chip technology in the framework of the PARTOX project funded by ANR in 2007. In PARTOX, cells were cultured inside tiny 'nanodrops' (100nl) which contain nanoparticles. Cells, from a lung strain, were cultured for three days (two days in contact with NPs), after which each cell from each drop was independently detected and analysed using several parameters. Some NPs appeared very toxic, (e.g., some rutile TiO2 NPs kill cells after 24 hours); however, most of them appeared not to be toxic in the acute phase. The partners were able to characterise the presence of an induced oxidative stress, for

example (measured on the 7-dichlorofluorescein fluorescent signal, DCHF). Interestingly, it was observed that the signal could be assessed on a cell-by-cell approach (Figure 1). These encouraging results demonstrated that it was possible to further miniaturise the cell culture system in order to reach a medium throughput assay (ideally 800 assays per chip). However, it was observed that culture conditions in the drops were not ideal since they may *per se* alter the phenotypes and render the interpretation of results to be complex.

The following conclusions could be drawn from this experience: i) further miniaturisation of the device is possible and will allow highly parallel integration; ii) data must be analysed from a single chip in order to compare the results at best; iii) the 100nl drops appeared not fully ideal as it was difficult to spot the cells in such a small volume; iv) impact of NPs can be evaluated on a cell-by-cell basis; v) physical and chemical analyses of NPs are mandatory to derive precise structure/function relationships; and vi) data flow is manageable.

The TOXDROP high throughput assay format has been considered for use with the comet assay. Unfortunately, the various requirements needed for an efficient comet assay led us to define new chips. They have been reported in the **FP6-COMICS** project report. Contact brigitte-s.fouque@cea.fr for more information.

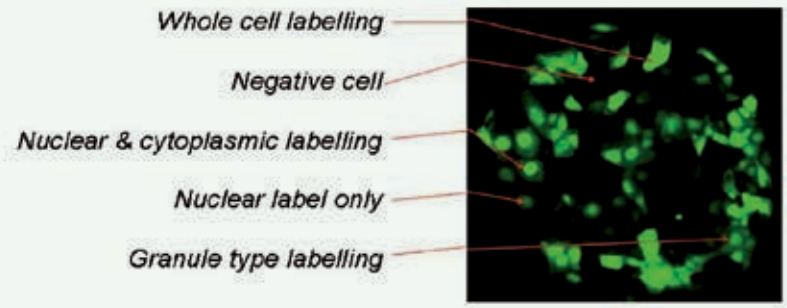
Figure 1. DCHF signal in 16HBE cells grown in 100nl drop on a 0.2mm2 hydrophilic spot.

Pathfinder™ technology follow-up

IMSTAR has extended its Pathfinder™ technology for high-content and high throughput screening to nuclear and cellular segmentation of different cell lines cultured in nanodrops. Based on fine morphological changes characterisation, this approach resulted in a novel and extremely powerful method to assess early toxicity of molecules present in the culture medium, far before classical oxidative stress reporters turn on. IMSTAR has extended its partnering with CEA Grenoble in the COMICS project (2007-2009) to empower the throughput and outputs of the comet assay on cell-on-chips. Contact info@imstarsa.com for more information.

Mass spectrometry Cell analysis follow-up

Within the STREP Project TOXDROP (2005-2006) methods were developed for direct imaging of molecules in single cells using Surface Mass Spectrometry (ToF-SIMS). Meanwhile, this technique shows now potential for direct imaging of xenobiotic materials in cellular environments. A German consortium will further exploit the possibilities for the detection and characterisation of nanoparticles in single cells. The project NanoPACT is in the final state of being admitted by the government of North Rhine-Westphalia. Contact daniel.breitenstein@tascon-gmbh.de for more information.



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COMICS

Comet assay and cell array for fast and efficient genotoxicity testing

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

Objectives

The Comics project aims to increase the throughput and speed of scoring of the well-established comet assay for DNA damage, so that it can be applied as a screening assay for genotoxic effects of chemicals (with cultured cells as a test system), as well as in biomonitoring studies with large numbers of lymphocyte samples. As well as strand breaks, damaged DNA bases are measured using lesion-specific endonucleases. New methods for studying DNA repair are also being developed both as variants of the comet assay, and as a chip-based assay. The high throughput versions of the comet assay are assessed for reproducibility in different laboratories, and their ability to detect effects of standard chemicals is compared with the original comet assay. SMEs are closely involved in the project, with the aim of developing hardware, image analysis software, customised enzymes, stains, etc. for marketing.

Experimental design

After preliminary exploration of various high throughput formats, the following were selected for further development, and are now being validated.

1. Agarose gels containing the cells to be tested for damage are set in a 12x8 array on sheets of GelBond. Four GelBond sheets can be run in one electrophoresis tank, giving a possible total of 384 samples in one experiment.

2. Twelve gels are set on one microscope slide, separated by a gasket so that incubations can be carried out independently. This format is particularly suited to the measurement of DNA repair activities in different cell extracts, and also to the use of different purified repair enzymes on cells

containing unknown damage. A total of 20 slides can be run in 1 tank (a potential total of 240 gels per experiment).

3. Cells are grown as mini-colonies on a specially treated glass slide, treated *in situ* as required, and then coated with agarose for comet assay processing. On parallel slides, cytotoxicity tests can be applied.

All methods require an advanced, rapid scoring method to replace conventional analysis of comets one by one. An automated image analysis system has been developed, based on the rapid accumulation of images for subsequent analysis.

Name of the test method	High-throughput comet assay, modified to detect specific DNA lesions, and to estimate DNA repair rate
Clinical endpoint	DNA damage (relevant to cancer initiation); DNA repair (reflecting individual susceptibility).
Cell (line)	In principle, any cultured mammalian cell line can be used, as well as white blood cells. For genotoxicity testing of chemicals, hepatocytes retaining xenobiotic-metabolising activity are most useful.
Method description	Cells embedded in agarose are lysed, treated with lesion-specific endonuclease, and subjected to alkaline electrophoresis. DNA loops containing breaks lose supercoiling and extend towards the anode, forming comet-like structures. To measure DNA repair, the cell extract is incubated with gel-embedded substrate DNA containing specific lesions.
SOP	Standard protocols are being devised in parallel with the new versions of the assay to ensure optimal performance.
Endpoints	The proportion of total DNA in the tail, visualised with a fluorescent DNA-binding stain, reflects DNA break frequency. In the repair assay, the rate of accumulation of breaks indicates the repair capacity of the cell extract.
How is a positive result defined?	Significant difference from negative control value.
How is a positive result expressed?	Increase in % tail DNA, or (after calibration) breaks per 109 daltons.
Applicability	Agents causing direct DNA damage, either strand breaks or altered bases: agents causing stimulation or inhibition of DNA repair.
Positive control	Cells treated with X-rays to induce breaks, or with photosensitiser plus light to induce base oxidation. In repair assays, known DNA repair endonuclease serve as controls.
Negative control	Untreated cells, with only the background level of DNA damage.
Performance	Sensitivity, specificity and accuracy are being assessed in a series of validation trials.

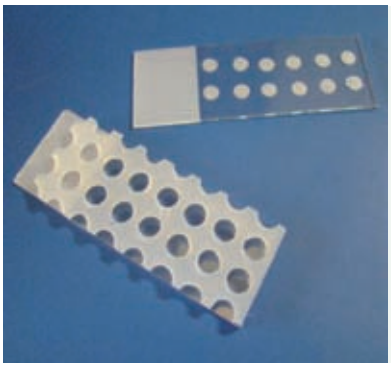
Name of the test method	High-throughput comet assay, modified to detect specific DNA lesions, and to estimate DNA repair rate
Can the test method be used in a regulatory safety context?	The REACH programme (testing chemicals for toxicity) is the most obvious opportunity for applying this technique.
Which R would the test method impact?	Reduction and replacement.
How can the test be used?	As part of an integrated alternative testing strategy.
Other important remarks?	The high-throughput approach has additional applications in the field of biomonitoring (measuring DNA damage and repair in lymphocytes).

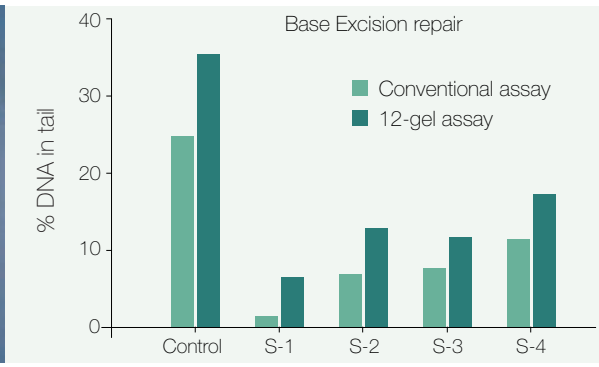
Results

The different high throughput designs have been fully developed, prototypes tested, and validation trials carried out, comparing the new with conventional comet assay methods. Figure 1 shows the 12-gel system, and results of an experiment measuring base excision repair rates. The new assay is at least as good as the conventional one at discriminating between different samples.

In the comet-based DNA repair assay, cells whose DNA contains either oxidised bases or ultraviolet light-induced pyrimidine dimers are embedded in agarose (on 12-gel slides), lysed, and incubated with cell extract, to measure respectively base excision repair (as in Figure 1) or nucleotide excision repair. In an alternative chip-based approach, an array of oligonucleotides with different DNA lesions serve as substrates for repair reactions with cell or nuclear extract. Several repair pathways can thus be examined in one single reaction incubation. The method has been used to define agents that inhibit DNA repair pathways.

Figure 1. Photograph of 12-gel comet slide, with gasket used to separate gels during incubation, and results of an experiment comparing this method with the conventional assay (2 gels per slide). Base excision repair activity was measured in lymphocyte extracts from four individuals (S-1 to S-4), with the enzyme formamidopyrimidine DNA glycosylase as a positive control.





Different staining methods are being compared. For instance, Figure 2 shows a comet stained by hybridising the DNA with fluorescent-tagged CotI DNA. This is the most repetitive DNA fraction and therefore hybridises uniformly over the whole genome. 'Padlock probes', specific for particular, unique DNA sequences, are also under development, and will be used to study gene-specific damage and repair.

For reference purposes, to test for inter-experimental or inter-laboratory variation, cells with DNA distinguishable from that of the test cells are included in the gel, as true internal standards. These reference cells contain a known frequency of DNA breaks and therefore serve as positive controls.

Several photosensitising chemicals have been synthesised, to induce specific oxidation damage in DNA. Dose response experiments have been carried out to establish their efficiency and specificity.

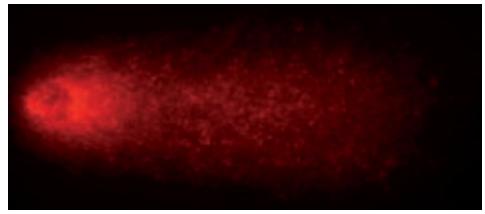
Figure 2. Alternative staining method for comets: Fluorescent in situ hybridisation of CotI DNA (highly repetitive sequences) to DNA of comet after alkaline electrophoresis.

Next steps

The potential for commercialisation of our novel methods is being carefully examined. We envisage a flexible, 'shopping basket' approach, allowing customers to select from a range of options – including hardware (tanks, gel-forming modules, etc.), lesion-specific enzymes, stains, and image analysis software.

Calibration and validation studies are continuing. Sensitivity will be assessed by carrying out dose response experiments with a range of standard chemical DNA-damaging agents.

Practical training courses, posters, and lectures at specialist workshops and conferences are planned, to bring our new methods to the attention of researchers in the fields of genotoxicity testing and biomonitoring.



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NanoTEST

Development of methodology for alternative testing strategies for the assessment of the toxicological profile of nanoparticles used in medical diagnostics

Grant agreement 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 essential for the risk assessment of these NPs. To be able to achieve this ambitious goal, the specific aims of NanoTEST are as follows: a) to carry out a detailed characterisation of selected NPs in order to define their main physico-chemical properties; b) to study specific and nonspecific interactions of NPs with molecules, cells and organs and to develop *in vitro* methods which can identify the toxicological potential of NPs; c) to validate *in vitro* findings in short-term *in vivo* models, to study manifestation of particle effects in animals and humans,

and to assess individual susceptibility in the response to NPs; d) to perform both Structure-Activity modelling and physiologically-based pharmacokinetic (PBPK) modelling of NPs; and e) to adapt the most advanced and promising assays for high-throughput automated systems and to prepare for validation by the European Centre for the Validation of Alternative Methods (ECVAM).

NanoTEST integrates the investigation of toxicological properties and effects of NPs in several target systems by developing a battery of *in vitro* assays using cell cultures, organotypic cell culture 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 JRC (ECVAM). Finally, we will propose recommendations for evaluating the potential risks associated with new medical NPs, which will be communicated to the scientific and industrial community.

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).
Clinical endpoint	Endpoints related to oxidative stress, inflammation, immunotoxicity, and carcinogenesis.
Cell (line)	We will 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. 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 are being modified or are under development. *In Vitro* Micronucleus Test will be performed following OECD 487 guideline, and the latest high throughput modifications of the comet assay developed within the COMICS project will be 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 will be 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: Fe3O4, and rare earth oxides, which are applied for contrast enhancement as well as for cancer treatment in various biomedical applications; c) metal fullerenes, which contain paramagnetic metals in the fullerene cage, proposed for use in MRI; d) polymeric materials utilised for the delivery of macromolecules (polylactic glycolic acid, PLGA; chitosan; hyaluronic acid), to be used as controls in order to ascertain the behaviour of NPs at the biological level; and e) quantum dots (QD), used as alternatives for organic dyes for cellular labelling, imaging and tracking.
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 TiO2. Certain tests have specific recommended positive control substances. A suggestion for the use of asbestos fibres or particles with similar carcinogenic effect is under consideration, at least for genotoxicity.
Negative control	Cells cultivated in medium in parallel with cells in medium with test-NP or positive control.
Performance	The aim is to develop assays with high sensitivity, specificity and accuracy, giving clear positive and negative predictive value. As results are not available yet, we cannot give more information than this.
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 fulfil 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.

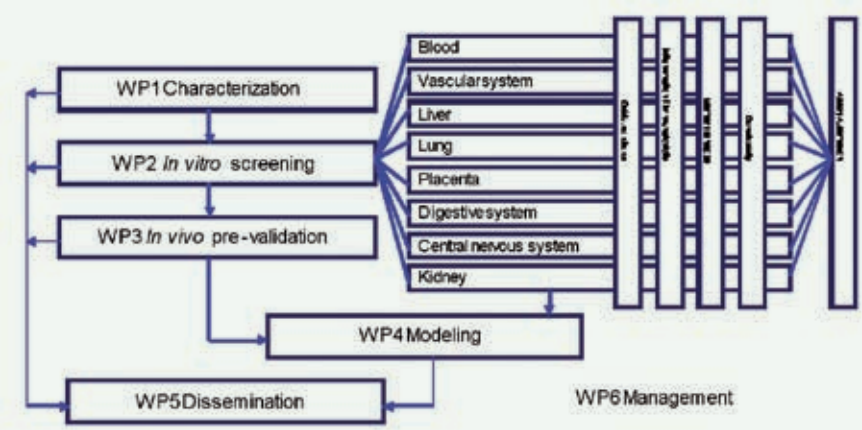

Figure 1. Overview of the project's activities.

Results

Results are not yet available as the project started only recently. The selection and characterisation of NPs has been performed and a first tier of assay candidates selected. A common template for database and reporting of results was agreed. All partners will use NapiraHUB database, which is modified from the Uclid 5 database developed for REACH.

Next steps

The toxicity of candidate nanoparticles will be tested using candidate assays and data will be included to NapiraHUB database. In parallel with *in vitro* assays, *in silico* methods will be developed. *In vivo* studies will be performed to validate *in vitro* results. 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 nanoparticles, which will be communicated to the scientific and industrial community.

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2.6 Forums and Workshops

Five projects have been working to promote the discussion and the acceptance of alternative testing methods and strategies, to establish the state of the art and to assess the gaps in knowledge in the research field under consideration.

CONAM, SCARLET, ForInVitox and InViToPharma are presented in this section, and START-UP is addressed in 'Defining the state of the art in 3Rs' section.

CONAM

Consensus networking on alternatives within Europe

Contract number: LSSB-CT-2004-504776
Project type: Specific Support Action (FP6)
EC contribution: € 150 000
Starting date: 1 March 2004
Duration: 36 months
Website: http://www.ecopa.eu

Objectives

The CONAM project was proposed by *ecopa* ('European consensus-platform for alternatives'), the only quadripartite not-for-profit organisation at EU level promoting the 3Rs strategy for the replacement, reduction, and refinement of animal experiments in research and regulatory testing. Consensus means that the four parties concerned, namely animal welfare, industry, academia, and governmental bodies, are represented in *ecopa* as well as in the individual National Consensus Platforms (NCPs), which form the building stones of the umbrella organisation.

The objective of CONAM was to build a solid extensive consensus network on 3Rs alternatives, ideally including all European countries (existing and new EU countries, as well as candidate countries), and with links to 3Rs-relevant organisations and institutes.

Other priorities of CONAM were:

- consensus networking on 3Rs alternatives with focus on website expansion, to support existing and new national consensus platforms, to stimulate collaboration and linking;
- ethics with emphasis on harmonisation and consensus by analysing and proclaiming shared ethical, legal and societal values, to continue dialogue where others failed and to develop consensus documents with focus on '-omics' technology and access to human data and samples;
- education activities to support (inter)national training and education in 3Rs methods, e.g. '-omics' technology, and to provide effective means of communication between the four parties.

- legislative issues with emphasis on the Chemicals Policy: to harvest relevant information on the translation of the White Paper into legislation, to disseminate and discuss it among the four parties, to come with a consensus paper to advise the EU decision making process.

One partner, VUB (Vrije Universiteit Brussel)/ ecopa, was involved in this project.

Approach

With regards to the creation of new platforms, *ecopa* organised, together with ECVAM, a stakeholder meeting in Prague in June 2004 to inform the new EU Member States about the 3Rs, as well as the validation of alternative methods and the work and objectives of *ecopa* (the minutes from this meeting are available for download at http://www.ecopa.eu/doc/PragueMinutes.pdf and the presentations are at http://www.ecopa.eu/inc/print.php?page=/content/archive/ecvam_04_presentations.php).

Within the CONAM project, contact building with relevant bodies and third parties has been realised, e.g. with the In Vitro Testing Industrial Platform (IVTIP), the OECD, the European Cosmetics Association (Colipa), the European Chemical Industry Council (CEFIC), the European Surveillance of Antimicrobial Consumption (ESAC), the European Technology Platform for Sustainable Chemistry (SusChem) and the European Partnership for Alternative Approaches to Animal Testing (EPAA), among others.

Results

CONAM has succeeded in doing the following.

1. In the field of networking:

- *ecopa*'s website was improved, expanded from a simple webpage to a full website where news, events, archive, education, databases, links and legislation sections were included;
- *ecopa*'s newsletter was created;
- Hungary and Poland established the NCPs *hucopa* and *polcopa*, respectively. Already existing organisations adapted their structure according to *ecopa*'s rules, so as to have representatives from the four parties concerned, e.g. Germany with the Stiftung set, and the United Kingdom with the Boyd group. New platforms were also launched, in Denmark, Ireland and Norway, for example, resulting in a total number of 16 NCPs (Austria, Belgium, the Czech Republic, Denmark, Finland, Germany, Hungary, Ireland, Italy, the Netherlands, Norway, Poland, Spain, Sweden, Switzerland, and the United Kingdom). Currently France is also a full member of *ecopa*, the UK has left, and of the 16 NCPs 2 are associates (Ireland and Poland).

2. In the fields of ethics:

- an ethics working group was formed which organised a consensus training course in Ljubljana in June 2005 – the abstract report is available on *ecopa*'s website (http://www.ecopa.eu/inc/print.php?page=/content/archive/slovenia_abstract_report.php);
- a workshop on 'The Use of Human Tissue' was organised in Brussels in November 2006 (more information is available at http://www.ecopa.eu/doc/ecopa_expert_workshop_20061124.pdf).

3. In the field of education and training:

- an educational working group was created and the Spanish platform REMA, being part of it, organised two training courses on

alternative methodologies in 2006, with a similar initiative taken on behalf of the Italian platform, IPAM, in Rome in February 2007;

- for the first time in Europe and working through *ecopa*, CONAM has created an information exchange system on alternative method development that almost in real time, supplies interested organisations, institutions and individuals with key information.

4. In the field of chemicals policy/REACH:

- a working group on 'Chemical Policy, REACH' was created, and besides the organisation of several workshops (e.g. in Brussels in February 2006, where discussions focused on the limitations and problems of REACH as regards the high number of animals used, and the implementation problems of alternatives), the working group also prepared a report on the impact of REACH on animal use (the workshop's programme is available at http://www.ecopa.eu/inc/print.php?page=/content/archive/reach_ws_05_programme.php, the presentations can be downloaded at http://www.ecopa.eu/inc/print.php?page=/content/archive/reach_ws_05_presentations.php, and more information is available at http://www.ecopa.eu/inc/print.php?page=/content/archive/reach_wg_statement.php and at http://www.ecopa.eu/inc/print.php?page=/content/archive/statement.php);

- a computer programme, 'The Animal Use Calculator', on the realistic number of animals used within the context of REACH was elaborated, and can be downloaded free of charge on *ecopa*'s website (available since 1 February 2006 at http://www.ecopa.eu/download.php?file=Animaluse_REACH_calculator.xls).

5. In the field of research:

- in order to stay updated with respect to developing research within the 3Rs, a working group on '3Rs-research' was created within the CONAM project, and as such, participation in several FP6 projects could be realised, e.g. in Predictomics, ReProTect, A-CUTE-TOX, Sens-it-iv, Biosim, Carcinogenomics, ForInvitox and Liintop.

Additionally, during the run of CONAM, an EU study was initiated by *ecopa* in collaboration with P&G and the Eurogroup for Animal Welfare on the availability of funding of alternative methods within the Member States, and other several workshops were organised as shown below.

- On annual basis, *ecopa* organised its plenary workshop in Brussels in November/December. These workshops always offered a forum for exchange of initiatives and experience with respect to alternative methods development. Each time, a special session was foreseen for young scientists to show their newest research results and the technologies involved (more information about these workshops is available at the 'archives' section of *ecopa*'s website).

- Within CONAM, the workshops '*ecopa* Science Initiative (eSI)' were organised on two occasions in Pueblo Acantilado in Spain, in October 2004 and September 2006. These workshops had the purpose of bringing young and promising scientists into contact with established researchers to discuss innovative technologies and their potential applications in *in vitro* research. Different scientific disciplines were combined in order to let scientists think 'about' and 'for' alternative methods. Some fruitful collaborative projects – focused on the further development and

optimisation of alternative methods – were initiated thanks to these workshops (more information about these workshops is available at the 'archives' section of *ecopa*'s website).

Publications

1. Rogiers, V., 'ECOPA: The European Consensus Platform on three Rs alternatives', Alternatives to Laboratory Animals (ATLA), 2004, 32, 349-353. Rogiers, V., 'Recent developments in the way forward for alternative methods: formation of national consensus platforms in Europe'. *Toxicology and Applied Pharmacology*, 2005, 207 (suppl. 2) S408-S413.

2. Rogiers, V., Pauwels, M., 'Good science must be the key factor in the development and use of alternative methods for safety assessment of cosmetics', *Altex - Alternativen zu Tierexperimenten*, 2005, 22 (Special Issue), 259.

3. Rogiers, V., Pauwels, M., 'Good science must be the key factor in the development and use of alternative methods for safety assessment of cosmetics', *Altex - Alternativen zu Tierexperimenten*, 2006, 23 (Special Issue) 346-352.

4. Rogiers, V., 'Replacement, reduction, refinement', *Public Service Review: European Union*, 2007, 13, UK, March Edition.

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SCARLET

Structure-activity relationships: experts' workshop in mutagenicity and carcinogenicity

Contract number:	LSHB-CT-2007-44166
Project type:	Specific Support Action (FP6)
EC contribution:	€ 112 840
Starting date:	1 June 2007
Duration:	12 months

Website: http://www.scarlet-project.eu

Objectives

The relationship between structure and toxic activity in chemical mutagens and carcinogens has been widely investigated. Structure-activity concepts have also been exploited to develop safer chemicals. While studies based on biased data sets may provide useful information for academic purposes, the extensive regulatory use of (Q)SARs foreseen by various legislative initiatives, including the REACH Regulation, requires that the models fulfil severe quality criteria. Unfortunately, this ideal situation is not a common occurrence in toxicology.

The determination of carcinogenic and genotoxic properties at present requires *in vivo* (carcinogenicity, some genotoxicity endpoints) and *in vitro* experiments that are time consuming, expensive and require a large number of experimental animals. (Q)SARs are an attractive alternative because they are fast, cheap and require no biological experiments. In fact, (Q)SARs are already routinely used for the risk assessment of chemicals by the regulatory agencies of some EU Member States, Canada, Japan, and the US, and the OECD has developed guidelines for their use.

Furthermore, the REACH Regulation identifies the need for increased information on the properties of chemicals, in order to ensure the appropriate management of their risks and hazards. To minimise animal testing, REACH is pursuing a smart and targeted testing strategy where predictions from (Q)SARs models can play an important role in filling data gaps and providing information for targeted bioassays.

SCARLET originates from a deep need in society to improve risk assessments for an increasing number of chemical compounds that is used in the modern world. It also relates to regulations, such as the requirements of the seventh Amendment of the Cosmetics Directive 76/768/EEC and of the REACH Regulation, to characterise the toxicological properties of a large number of chemicals.

This project aimed at organising a discussion and a workshop so that leading experts could critically review mutagenicity and carcinogenicity in the field of (Q)SARs, while identifying important issues that require further investigation.

The application of (Q)SARs in a regulatory setting is still controversial. Therefore, SCARLET project's partners have identified the following specific objectives for the workshop:

identification of requirements for (Q)SARs models for genotoxicity and carcinogenicity from the perspective of various stakeholders (scientists, regulators, end-users and industries);

evaluation of existing (Q)SARs models and techniques in respect to these requirements and to the OECD principles for validating (Q)SARs models;

identification of open research issues and harmonisation of efforts to solve them;

preparation of a document with recommendations for the regulatory application of genotoxicity and carcinogenicity (Q)SARs containing, for example, available databases, proposed protocols, criteria for performance acceptability and suitable software characteristics.

These societal objectives generate a series of scientific objectives. The key issues have already been addressed by the 'OECD Principles for the Validation of (Quantitative) Structure-Activity Relationship Models for Regulatory Purposes' but the guidelines do not provide objective acceptance or rejection criteria for (Q)SARs models.

In addition, it is likely that the stakeholders will require additional criteria for the acceptance of (Q)SARs models. For this reason, the main scientific and technical objective was to specify the OECD and stakeholders' requirements, as well as to establish unambiguous and objective acceptance criteria for genotoxicity and carcinogenicity predictions. Based on the requirements of the stakeholders, it was anticipated that further scientific or technical topics would have to be addressed, including the following.

Proposing a platform for organising knowledge about databases, algorithms and programmes that are suitable for modelling genotoxicity and carcinogenicity endpoints;

Proposing a framework for the integration of multiple (Q)SARs studies that deal with their complexity and redundancy. Both statistically and mechanism-based approaches should be used and ideally combined; the results have to be validated (proof of principle).

Evaluating the role of (Q)SARs tools within the framework of an intelligent testing strategy for a decision support system (e.g. by integrating *in vivo*, *in vitro* and *in silico* data, taking into account the experience in Europe and the US).

Identifying unresolved research issues within this area and coordinating research efforts.

Approach

SCARLET was a multidisciplinary and multisectorial project, addressing regulators, scientists, problem holders, animal welfare experts, citizen organisations, and the chemical industry.

At the SCARLET workshop, which took place in Milan, April 2008, 52 participants from 11 countries were present, combining the experience of the various scientific communities working in this area. The participants evaluated the current state of the art, identified open research issues, discussed a framework for comparing and integrating individual research activities and recommendations on the use of computer-based methods in carcinogenicity, mutagenicity and genotoxicity, and provided scientific guidance

for the implementation of genotoxicity and carcinogenicity (Q)SARs for regulatory purposes.

The objectives listed before have been addressed within the workshop, in the preparatory discussions of the advisory committee, and by the use of a questionnaire for the stakeholders; a summary of the workshop's discussion and other material, such as the poster presentations, are available at SCARLET's website (http://www.scarlet-project.eu). The website also includes links and descriptions about software and databases of interest related to the topic.

Results

SCARLET workshop's discussion started with an overview of the new *in vitro* experimental approaches. A major issue is the occurrence of false negatives and positives. The precautionary principle is pointing towards a conservative approach, which tends to eliminate chemicals with positive results, but the use of batteries of *in vitro* methods is progressively eliminating most of the compounds, with an increasing number of false positives.

Nevertheless, *in vitro* methods are used for regulatory purposes, and they are required information within many evaluation protocols and laws. It was mentioned that when the Ames test was originally introduced, it was severely criticised as not being suitable to replace carcinogenicity studies. Today it is widely accepted that mutagenicity studies are a valid support to the general evaluation of the genotoxicity studies. It is expected that a similar evolution will also happen for *in silico* methods. Indeed, there is a knowledge gap on the carcinogenicity and mutagenicity evaluation of the chemical compounds. Any useful piece of information should be used to limit toxic effects. Alternative methods, such as *in vitro* and *in vivo* methods, can contribute to cover this knowledge gap.

The positions of industry and the regulation frameworks

At the workshop, the positions of industry and regulators were discussed. Pharmaceutical companies, chemical, cosmetics, and food industries have needs, which are partly similar, partly different. Different regulations apply, which vary in different countries. In Europe, the regulation for cosmetics is progressively banning all animal experiments. Thus, only alternative methods will be used. The situation for pharmaceutical companies, which will continue using animals, is different. The REACH regulation is promoting the use of methods alternative to animal models. However, their reliability has to be proved.

Furthermore, in the US, certain *in silico* models are used when providing data for the Food and Drug Administration, while they are not so popular in Europe.

Regulators and industry may not pay the same attention to false negatives (of high concern for regulators) and false positives (which cause the banning of certain compounds with industrial interest, but no toxicity). Furthermore, the overall evaluation of a certain chemical compound may be different in the different industrial sectors, considering for instance the balance between the risk and the benefits, or if the compound is a natural food component.

The different in silico *methods*

More and more *in silico* methods are appearing. They differ for the theoretical basis and for the purposes. Thus it is not appropriate to evaluate them with a single perspective. Conversely, the practice in several industries is to adopt a battery of *in silico* tools.

Several commercial/public software and databases are available, and a number of methods have been published in the literature. Some methods are based on the human expert

knowledge, codified into rules, while other methods are based on knowledge engineering techniques. Some methods use chemical descriptors, others fragments, in particular active sub-structural components. Some methods predict toxicity categories, while others predict the toxicity potency. Some methods are global (ideally useful for a large set of compounds, even if restrictions exist), and others apply to a much more specific skeleton/chemical class.

The accuracy of the models for mutagenicity is higher, around 75% to 80%, while for carcinogenicity it is lower (65%).

It is important to consider the reproducibility of the experimental methods (for instance, for mutagenicity it is 85%).

The purposes of the model can be different, and thus its evaluation procedure. For screening/ prioritisation purposes, all methods can be useful, provided that they enrich a certain component (for instance, they identify many of the genotoxic compounds). For a more precise evaluation, stricter criteria apply, and many methods may not be suitable.

The perspectives

In such a broad scenario, with different components, complex, multiple solutions exist that are not only theoretical, but also related to the application and use. The challenge is to tackle the problem in its complexity, taking advantage of the new tools offered by new assays and the omics techniques. The concept of toxicity profiles has gained new prominence. *In silico* methods can benefit from this increased information, and better contribute to carcinogenicity studies.

Other results

A list of suitable software (Table 1) and databases (Table 2) was compiled (also available at SCARLET's website) and a scientific paper has been published in the *Journal of Environmental Science and Health,* Part C [1].

1 Benfenati, E., Benigni, R., DeMarini, D.M., Helma, C., Kirkland, D., Martin, T.M., Mazzatorta, P., Ouédraogo-Arras, G., Richard, A.M., Schilter, B., Schoonen, W.G.E.J., Snyder, R.D. And Yang, C., 'Predictive Models for Carcinogenicity and Mutagenicity: Frameworks, State-of-the-Art, and Perspectives', *Journal of Environmental Science and Health,* Part C 2009;27:57-90.

Table 1

Software	Brief description and website	Website
Leadscope (commercial)	Data Management and Decision Support.	http://www.leadscope.com
ACD/Chemfolder (commercial)	Database Management Software for Chemical Structures, Chemical Properties and Experimental Data.	http://www.acdlabs.com/
PowerMV (freeware)	Application that can used to import, view, edit, print and export SDF files. This application also offers statistical analysis, dynamic molecular viewing, descriptor generation, and similarity search analysis.	http://www.niss.org/PowerMV/
Toxtree, European Chemicals Bureau (ECB) (freeware)	Application that places chemicals into categories and predicts various kinds of toxic effect by applying decision tree approaches, such as the Cramer classification scheme, the Verhaar scheme for aquatic modes of action, rule bases for skin and eye irritation and corrosion, and rule base modules for mutagenicity and carcinogenicity prediction.	http://ecb.jrc.it/qsar/qsar-tools/index.php?c=TOXtrEE
Oncologic (freeware)	Program that evaluates cancer-causing potential by applying structure activity relationship (SAR) analysis and mimicking the decision logic of human experts.	http://www.epa.gov/oppt/newchems/tools/oncologic.htm
OECD QSAR Application Toolbox (freeware)	Software tool that allows the user to do the following: (a) make (Q)SARs estimations for single chemicals; (b) receive summary information on the validation results of the model according to the OECD validation principles; (c) receive a list of analogues, together with their (Q)SARs estimates; (d) receive estimates for metabolite activation/detoxification information.	http://www.oecd.org/env/existingchemicals/qsar
Toxmatch ECB (freeware)	Open-source software application that encodes several chemical similarity indices to facilitate the grouping of chemicals into categories and read-across.	http://ecb.jrc.it/qsar/qsar-tools/index.php?c=TOXMATCH

Software	Brief description and website	Website
DART ECB (freeware)	(Decision Analysis by Ranking Techniques) is a software tool designed for the ranking of chemicals according to their environmental and toxicological concern based on the most recent ranking theories.	http://ecb.jrc.it/qsar/qsar-tools/index.php?c=DART
HazardExpert (commercial)	Rule-based expert system for toxicity estimation. Predicts the toxicity of organic compounds based on toxic fragments.	http://www.compudrug.com
DEREK for Windows (commercial)	Expert knowledge base system for toxicity prediction.	http://www.lhasalimited.org/
TOPKAT (commercial)	An expert system of statistically derived models for toxicity prediction.	http://www.accelrys.com
MultiCASE (commercial)	Toxicity prediction via identification of molecular substructures relevant for the observed biological activity.	http://www.multicase.com/
TIMES (Tissue Metabolism Simulator) (commercial)	Toxicity prediction of chemicals resulting from their metabolic activation. Skin sensitisation and mutagenicity models.	http://www.oasis-lmc.org
MetabolExpert (commercial)	Program for prediction of the metabolic fate of a compound in humans, animals or in plants.	http://www.compudrug.com
Bioloom (commercial)	Calculations of hydrophobic and molecular refractivity parameters.	http://www.biobyte.com/bb/prod/bioloom.html
Episuite US EPA (freeware)	Physical / chemical properties and environmental fate estimation models.	http://www.epa.gov/oppt/exposure/pubs/episuite.htm
PharmaAlgorithms (Commercial)	Software tools for molecular discovery in pharmaceutics and biotechnology.	http://pharma-algorithms.com/
Pass (Commercial)	Prediction of Activity Spectra for Substances.	http://195.178.207.233/PASS/

Table 2

Databases	Brief description	Website
ESIS (European Chemical Substances Information System) ECB	IT System that provides information on chemicals.	http://ecb.jrc.it/esis/
TOXNET US NIH	Cluster of databases on toxicology, hazardous chemicals, environmental health, and toxic releases.	http://toxnet.nlm.nih.gov/
DSSTox (Distributed Structure-Searchable Toxicity) Database Network US EPA	Public database network of downloadable, structure-searchable, standardised chemical structure files, associated with toxicity data.	http://www.epa.gov/ncct/dsstox/index.html
The Carcinogenic Potency DataBase (CPDB)	Standardised analyses of the results of 6 540 chronic, long-term animal cancer tests.	http://potency.berkeley.edu/
The Pubchem Project US NIH	Provides information on biological activities of small molecules.	http://pubchem.ncbi.nlm.nih.gov/
Danish EPA (Q)SAR Database	Repository of estimates from over 70 (Q)SARs models for 166 072 chemicals. The (Q)SARs models encompass endpoints for physicochemical properties, fate, eco-toxicity, absorption, metabolism and toxicity.	http://ecb.jrc.it/qsar/qsar-tools/index.php?c=ddb
US National Toxicology Program (NTP) - Study Reports	Study reports on toxicological evaluations of substances of public health concern, performed under NTP's aegis.	http://ntp.niehs.nih.gov/
International Agency for Research on Cancer (IARC) Monographs	Critical reviews and evaluations of evidence on the carcinogenicity of human exposures.	http://monographs.iarc.fr/
Survey Of Compounds (Commercial)	Electronic database that collects the contents of all published volumes of U.S. Public Health Service Publication No. 149, 'Survey of Compounds Which Have Been Tested for Carcinogenic Activity'.	Not Available
C-QSAR (Commercial)	Database of over 14 000 (Q)SARs, divided in two major chemistry areas: biological and physical-organic.	http://www.biobyte.com/bb/prod/cqsarad.html

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ForInViTox

Forum for researchers and regulators to meet manufacturers of toxicology test methods

Contract number: LSSB-CT-2007-037779
Project type: Specific Support Action
EC contribution: € 288 850
Starting date: 1 September 2007
Duration: 24 months
Website: http://www.forinvitox.org

Objectives

With the support of the European Commission, researchers and the industry in recent years have conducted research on *in vitro* replacement tests. This has resulted in an important number of scientifically sound methods and new strategies, but the transfer of these inventions to potential users has been much slower than expected, mainly due to difficulties encountered in the transferability and official approval, as well as the production of test kits under conditions that meet the requirements of good laboratory practice (GLP).

In order to ensure that research has the desired socioeconomic impact, the present gap between inventions and potential users needs to be bridged. The purpose of the ForInViTox project is to identify the bottlenecks in the transfer process and establish a forum where representatives of manufacturers, research projects and regulatory agencies, as well as end-users of *in vitro* or *in silico* methods continuously get a chance to discuss how to speed up the process of making *in vitro/in silico* methods available for commercial use.

Approach

The project is structured into six work packages (WPs). Three are devoted to retrieving the relevant information required: availability of inventions (WP1); present and what will presumably be future needs of users (WP2); and ability of currently existing manufacturers (WP3) to meet the required demands. With the aid of experts, an analysis of the outcome of these three exercises was followed (WP4). The ultimate goal of the project is to set up a forum where the interested parties can meet (WP5). The last work package (WP6) includes the overall management.

Results

WP1 focused on retrieving information from 'inventors' developing *in vitro/in silico* methods from identified FP6 projects. A questionnaire was designed including questions about endpoints and methods that are at different stages of development (basic research, in-house validated, in validation) and was submitted to the coordinators of the FP6 projects. It was concluded from the answers that there is a number of scientifically available methods and that the transfer of these to potential users' needs to be further explored.

Among the difficulties for commercialising test systems, some coordinators considered that they have not properly explored product commercialisation. Different expectations of inventors and companies on the outcome of the exploitation are also mentioned. Lack of reproducibility and a low degree of quality control were not considered as major problems. However, one coordinator mentioned big inter- and intra-laboratory variability.

WP2 was in charge of the identification of the needs of users. A questionnaire was sent to IVTIP (In Vitro Testing Industrial Platform) members as well as other companies. The conclusion from the responding users was that the companies have adapted to the existing methods and have learned how to use them to make their decisions. They do also feel that more mechanistic, multi-parametric and physiological models of human origin are required. They emphasise the importance of very robust and reproducible assays, therefore commercialisation under GMP could be a potential solution.

The majority of the companies highlighted that *in vitro* models are already part of the strategy of characterisation and safety of new chemicals. Most of the times, the answer indicates that they use a single end point (like cytotoxicity). Although it is recognised that cytotoxicity is poorly mechanistic, it is considered as sufficient for the expectations posed in the assay. Nevertheless, the results obtained have a limited impact on the corporate final decision mainly due to the lack of guidelines.

Finally, a series of new approaches were proposed that could be summarised in three aspects that should be improved: (a) the need to increase complexity by the use of multimetric end-points into the simple cellular models; (b) incorporate complex models in the testing battery; and (c) implement more 'human' testing models, more related to the final target.

The aim of **WP3** has been to produce an inventory of companies in Europe that are or could become involved in the production of alternative toxicity tests, and to learn about their strengths and weaknesses. A questionnaire with questions about their organisation, testing capacity, quality controls, and method(s) in production was sent out. Among the responding companies the most common toxicological area was for genotoxicity/mutagenicity testing, and the largest group of customers reported was the pharmaceutical industry. It was concluded that a big problem for technology transfer is the lack of intra- and inter-laboratory reproducibility, as well as the lack of regulatory and scientific acceptance for alternative tests.

The basic requirement needed for an invention to be further developed into a commercial product is that it be reproducible. It should also comply with regulations, be practical, low cost, and have screening capacity. In addition, the quality controls need to be improved, the complexity of the assays/models must be solved – more simple and robust assays are needed, as well as reference compounds and correct training of personnel on how to use the assay/model. Further, it is also important to speed up the validation procedures and to increase cooperation between producers, users and researchers of alternative toxicity tests to meet the future demands of true replacements for *in vivo* tests.

The information from the three inventories were summarised (**WP4**) and the **general conclusions** are the problems and difficulties with intra- and inter-laboratory reproducibility and the lack of acceptance for *in vitro*/*in silico* toxicity tests. Further, it is also important to improve the quality controls for the tests, and to increase cooperation between producers, users and researchers of alternative toxicity tests.

From the analyses made in **WP1 through WP3,** a list of relevant subjects was put together for the discussion at the expert meeting in Stockholm in June 2008. The purpose of the expert meeting was to give an analysis of the retrieved data, and to identify the problems, questions and bottlenecks in transferring *in vitro* methods from the early stage of research innovations to commercial use by the end-users. These results were used to propose actions and specific goals for the first Forum Event that took place from 12 to 14 May 2009 at the Karolinska Institute in Stockholm. The programme of this workshop can be found at http://www.forinvitox.org/docs/the-forum-event-program-23-april.pdf and it is also presented in the Annex to Invitopharma's text, in this book. The Expert meeting came to the conclusion that there is an intellectual, cultural, financial, and legal gap between the inventors and the final users that needs to be identified and bridged. To further improve the technology transfer of *in vitro* tests, the development process should be reformed, but it is also important to overcome the scientific, economical commercial, technical, political as well as legal obstacles for transfer of *in vitro* methods today.

Essential parts for the transformation of an invention into a commercially applicable test method are the transformation to a less complex method, robustness of the method, GLP, validation, different competence for the different steps of the process, and the early involvement of regulators. Instruments to facilitate the transfer were identified as clear guidance and criteria for validation, showing good and bad examples of technology transfer, a cookery book for the steps of the transfer process and matching inventions with applications.

A Forum website (http://www.forinvitox.org) was set up in January 2008. This virtual forum offers a possibility for producers to present their products.

Next steps

The outcome of the Forum Event that took place in Stockholm in May 2009 will be a White Book recommending actions to meet the increased needs for alternative toxicity tests.

Partners

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InViToPharma

Workshop on the need for *in vitro* toxicity tests within the pharmaceutical industry

Contract number: LSSB-CT-2007-037814
Project type: Specific Support Action
EC contribution: € 578 000
Starting date: 1 November 2007
Duration: 14 months
Website: http://www.forinvitox.org/invitopharma

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 the 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 monitoring the toxicological mechanism in detail, etc.;
- to identify and present academic model systems suitable for use by 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.

Approach

The project started with a general analysis of the supply of knowledge and products that can be used within the preclinical testing and the corresponding demand for efficient and robust *in vitro* tests from the European pharmaceutical industry. The information that formed the base for the analysis was retrieved through questionnaires to pharmaceutical companies.

A two-day workshop on the subject 'What is the need of *in vitro* toxicity tests within the pharmaceutical industry' took place at Silverdal Science Park in Stockholm, Sweden. The workshop comprised both introductory lectures and smaller working groups focused on different issues defined after the general analysis mentioned before. The outcome from the working group meetings will be reported at a session for all participants followed by a general discussion. A final report from the workshop will be produced and distributed.

Results

The following inventories took place during the first 12 months 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;
- a report summarising the current situation;
- the establishment of collaborations with the 'New Safe Medicines Faster' initiative, 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. This Forum Event took place between 12 and 14 May 2009 at the Karolinska Institute in Stockholm. The programme of this workshop can be found at http://www.forinvitox.org/docs/the-forum-event-program-23-april.pdf and is presented in the Annex. The meeting was a joint event of the EU-projects Forinvitox and Invitopharma and of The In Vitro Testing Industrial Platform, and was organised by Silverdal Science Park in 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 presented are the best positioned to become future *in vitro* test methods with commercial potential.

The potential impacts of the Forum Event are:

- enhancement of the links between researchers in the *in vitro* field and technology users;
- contribution to future less expensive and more reliable drug safety testing in alignment with the three Rs principle;
- contribution to an increased competitive power of the European pharmaceutical industry by facilitating the development and use of *in vitro* models and tests in drug development and safety testing;

- Opportunity for the manufacturers of *in vitro* tests to present their products and to make personal contact with representatives from the European pharmaceutical companies.

Next steps

There will be a report produced that summarises the presentations and the discussions during the Forum Event. A draft of the final report will be distributed to the members of the Advisory committee for comments that will be considered in the published version. The most effective channels to distribute the report will be discussed with the advisory committee.

Partners

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Programme for the Forum Event – market place for *in vitro* tests

Karolinska Institutet, Stockholm, Sweden 12-14 May 2009

12 May

13.00 **Opening session of The Forum event of Forinvitox and Invitopharma**

Erik Walum, Board member of Silverdal Science Park, Sweden
Jürgen Büsing, Project Officer, EC (to be confirmed)
Welcome presentations

Erica Toft, Silverdal Science Park, Sweden
Antek Wielburski, Expertrådet, Sweden
Introduction to the Forum event: Summary of the Forinvitox and Invitopharma projects

13.40 **Session 1: Available *in vitro* methods with potential to be commercialised**
Chair: Erik Walum

Oliver Engelking, CellSystems® Biotechnologie Vertrieb GmbH, Germany
Human Reconstructed Skin – a success story

14.00 **Meeting/Market time**
Poster session, Exhibition, Match making activities

14.30 **Oral presentations of *in vitro* methods and a panel commenting the methods (I)**

Jef Hooyberghs, Greet Schoeters, Rosette Van Den Heuvel
VITOSENS™: a cell-based alternative to identify skin sensitizers by gene expression

Rodger D. Curren and Marilyn J. Aardema
Development of a New *In Vitro* Genetic Toxicology Model Using Reconstructed Human Skin

Anna Forsby, Johanna Lilja and Heléne Lindegren
The *in vitro* nociception assay for estimation of mild eye irritation

15.30 **Meeting/Market time**
Poster session, Exhibition, Match making activities

16.15 **Oral presentations of *in vitro* methods and a panel commenting the methods (II)**

David Rozzell, Moo-Yeal Lee, Jessica D. Ryan, Jonathan S. Dordick, Douglas S. Clark
Miniaturized Three-Dimensional Cell Culture and Metabolic Enzyme Arrays For High-Throughput Toxicity Assays

Dieter Runge and Anett Ullrich
HEPAC^2: Serum-free, standardized and validated (re-usable) primary human hepatocytes for the analysis of xenobiotics

Maya R.Vilà, Agustín Lahoz, Laura Picazo and Myriam Fabre
ISOCYP-TOX: A new ready-to-use concept for *in vitro* evaluation of biotransformation-mediated toxicity

17.15 **Meeting/Market time**
Poster session, Exhibition, Match making activities

17.45 **Oral presentations of *in vitro* methods and a panel commenting the methods (III)**

Samuel Constant, Song Huang, Jean Paul Derouette, Mireille Caulfuty
The use of an *in vitro* cell model of the Human Airway Epithelium (MucilAir™) in preclinical development

Marianna D Gaça, David Thorne, Jason Adamson, Eian D Massey
A model of *in vitro* exposure to aerosols at the air-liquid interface

18.15 **General discussion**
Summing up the day: questions suggested by participants and end with question "do the offers fit with the demand?"

20.00 **Dinner/Get together party at Karolinska Institutet**

13 May Bridging the gaps between the inventors and final users

8.30 **Session 2: Testing needs and expectation - managed by IVTIP**
Chair: Bart de Wever

Bart De Wever and Erwin Roggen, IVTIP In Vitro Testing Industrial Platform, Belgium
Bridging the gaps between inventors and final users
Testing needs and expectations: the importance of industrial applicability

Klaus Schröder, Henkel AG & Co, Germany
The application of the 7th amendment: what models for what use?
(Title to be announced)

Gavin Maxwell, Unilever, UK
Aspects on technology transfer of *in vitro* methods (Title to be announced)

10.30 **Meeting/Market time**
Poster session, Exhibition, Match making activities

11.15 **Oral presentations of *in vitro* methods and a panel commenting the methods (IV)**

Joachim Wiest1, M. Brischwein[2], A.M. Otto[3] and B. Wolf[2,3]
Label-free cell based assays as alternative testing method for toxicity

Sundeep Bhandari
Nikon BioStation CT– an 'all-in-one' toxicity testing system solution

José-Enrique O'Connor, Guadalupe Herrera, Laura Díaz and Emilio Barberá-Guillem
The Use of PetakaG2™ Hermetic Cell Culture Containers and Cytomics for Testing of Sustained *In Vitro* Toxicity (MultiCytox-LT®)

12.30 **Meeting/Market time**
Poster session, Exhibition, Match making activities

13.00 **Lunch**

14.00 **Session 3: Acceptance and validation - The regulators point of view**
Chair: Odile de Silva/Lawson, L'Oreal, France

Matthias Peiser, Federal Institute for Risk Assessment, Germany
(Title to be confirmed)

Representative from DG Environment (to be confirmed)

Cosmetic registration and 7th amendment to the cosmetic directive: the regulator point of view, (speaker to be confirmed)

15.30 **Meeting/Market time**
Poster session, Exhibition, Match making activities

16.15 **Oral presentations of *in vitro* methods and a panel commenting the methods (V)**

Manuela Dahinden-Hase, Jean-Baptiste Fini, Vincent Laudet and Ingemar Pongratz
SME RECEPTOR – an industry-academia partnership for *in vitro* toxicology testing

Matthew D. Rand
A Drosophila embryo platform for high-throughput testing of suspected toxins and bioactive compounds

Cristina Suñol and Anna Forsby
Combination of cell based assays determining GABAA receptor activity and Cell Membrane Potential for *in vitro* testing of neurotoxicity

Dorothée Hallier-Vanuxeem, Lucie Dehouck, Emmanuel Sevin and Roméo Cecchelli
Ready-to-use *in vitro* Blood-Brain Barrier model

18.00 **Session 4: Technology transfer**
Chair: Erica Toft

Claes Post, Karolinska Institutet Innovation, Sweden
(Title to be announced)

Märit Johansson, Karolinska Institutet Science Park, Sweden
(Title to be announced)

Jonas Ekstrand, Actar, Sweden
(Title to be announced)

20.00 **Dinner at Haga Park**

14 May

9.00 **Session 5: Quality and scientific acceptance of alternative methods**
Chair: Gavin Maxwell

Sandra Coecke, ECVAM, Italy
(Title to be announced)

Hans H. Lindén, EUFEPS, The European Federation for Pharmaceutical Sciences, Sweden
(Title to be announced)

Rodger D. Curren, IIVS, Institute for In Vitro Sciences, USA
Human tissue models used for safety testing: scientific, economic and regulatory considerations

Discussion around points to consider when moving towards acceptance initiated by
Erin H. Hill, Institute for In Vitro Sciences, USA

11.00 **Meeting/Market time**
Poster session, Exhibition, Match making activities

11.30 **Panel discussion**
General questions and aspects with the final question: How useful was the Forum Event?

12.15 **Conclusions and closing of the Forum Event**

12.30 **Lunch**

3. FUTURE STEPS

3.1 Defining the state of the art in 3Rs

In order to define strategies for future research work and the respective RTD priorities, a clear picture of the current state of the art and existing gaps in knowledge is a prerequisite.

The *Specific Support Actions* funding scheme of the Framework Programme, for training, conferences and prospective studies in support to the programme, is thus the suitable tool to supply the Commission with the necessary scientific support.

START-UP is a currently ongoing Specific Support Action project which will contribute to the identification of future research priorities in the field of 3Rs for the process of drug development with a series of structured workshops and the corresponding road map.

START-UP opens up a new kind of alternative test-need identification and accordingly solutions in the following way: 'ask the real experts within industry, not visible in the public 3R-scene, in closed expert rounds and have a balanced NGO to get those real issues identified. Then, take these issues up, in a kind of "debate mode" workshops to come to a realistic road map for further, well-planned work to be done in the field of 3R alternatives'.

As an initial step of the START-UP Project, some of the 3R gaps in pharma could already be identified.

Refinement of animal disease models in research fields of CNS-disorders, rheumatic diseases, diabetic mellitus, cardiovascular problems.

Directing the development of transgenic models to real industrial needs, leaving no space anymore for scientific playing around.

More careful evaluation of the relevance of the 'to be validated' alternatives and the promises made with respect to their potential value in pharma applications.

How useful and revolutionary are *in vitro* models mimicking the blood brain barrier, for example? Do they fill a real industrial need or are these just being developed without a clear purpose and applicability domain?

How good is QSAR, and is it really an under-appreciated modelling technique, existing for many years, but 'only now being rediscovered' by the pharmaceutical industry?

Calls for proposals need a new basis, built on needs seriously identified and not yet covered by existing initiatives and partnerships such as the Innovative Medicines Initiative (IMI), the European Partnership for Alternative Approaches to Animal Tests (EPAA), and others. Indeed, the question may be posed on how complementary, respectively overlapping these different partnerships/networks are.

The pre-meetings of the START-UP Project point towards different areas of research, areas that are not typically classified as alternative methods research fields, but that are providing a definite win-win situation for animal welfare, alternative method introduction and industrial performance.

The START-UP project is now entering into its second and final year with workshops on each of the 3Rs, and is making use of the wealth of information gathered during the closed pre-meetings coming from pharmaceutical industry experts and also excellent young scientists.

Recommendation for meaningful 3R research will be worked out, and soon an up-to-date road map will be made available to the Commission.

Contribution based on private communication with Prof. Vera Rogiers, Coordinator of START-UP, April 2009.

START-UP

Scientific and technological issues in 3Rs alternatives research in the process of drug development and Union politics

Grant agreement number: HEALTH-F5-2007-201187
Project type: Specific Support Action (FP7)
EC contribution: € 317 964
Starting date: 1 April 2008
Duration: 24 months
Website: http://www.ecopa.eu

Objectives

The START-UP project aims to identify, and make proposals to eliminate, bottlenecks in the 3Rs approach in pharmaceutical discovery and development. To this end, the project will organise three workshops to determine the state of the art of the 3Rs in the EU, assess strengths and gaps and identify rate-limiting steps on a scientific and technological level. This will lead to a consensus paper containing concepts and suggestions for a road map for future research.

Stakeholders (among them European pharmaceutical industries) have identified bottlenecks in drug development and in the integration of *in vitro* methods. Early identification of wrong or under-performing candidates is essential for the competitiveness of the European industry. Identification of bottlenecks in the implementation of the 3Rs in drug research and development should help identify the best *in vitro* and *in vivo* systems, as well as speed up the drug development process. Existing hurdles at the scientific, technological, ethical, regulatory and political levels play a substantial role and are rate limiting in developing new drugs, including new biological entities.

Approach

The project is structured around three workshops, one for each R, preceded by three expert meetings re-defining and prioritising current bottlenecks in the 3Rs methodology, drug discovery and development. The limitations and gaps of each phase are addressed, e.g. the majority of the currently used cell systems does not have the required stability for genomics, proteomics or metabonomics and often lack crucial bioactivation capability. Thus, the status of satisfactory 'predictive' pharmacology and toxicology *in vitro* has not been reached yet. The final goal is a consensus document in which a road map for implementing the strategy for

a better integration of 3Rs in the EU drug discovery and development strategy is proposed.

Results

ecopa (European Consensus Platform on 3R Alternatives) organised three Expert Meetings with:

1. the pharma industry and regulatory bodies on bottlenecks in the drug discovery and development process (19 and 20 May 2008, in Madrid, Spain);

2. experts in animal disease models of the pharma industry (5 September 2008, in Basle, Switzerland);

3. young researchers and established top-level scientists on emerging *in vitro* technology (16-18 October 2008, in Alicante, Spain).

The information gathered from the Experts Meetings is being loaded into three workshops, one for each R, organised by *ecopa*'s members, namely the National Consensus Platforms.

The first workshop already took place (26 and 27 February 2009, in Rome, Italy) and recommendations on Refinement are being worked out. The programme of this workshop can be found at http://www.ecopa.eu/doc/START-UP_Workshop_Refinement_Final_Programme.pdf and is presented in Annex.

Next steps

The next steps are the organisation of the workshops on Reduction (in Innsbruck, Austria) and Replacement (in Budapest, Hungary), and making appropriate recommendations in these fields. All this information will be critically analysed to finally come to an expert 3Rs report and a road map for further research.

Partners

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3R eplacement
eduction
efinement

ecopa

Refinement: bottlenecks in pharmaceutical investigations

Roma 26-27 February 2009
Istituto Superiore di Sanità, Aula Bovet
Viale Regina Elena 299
Workshop organized by IPAM, FINCOPA, POLCOPA

Scientific Committee
Franca Fassio, Bernward Garthoff, Peter Maier, Vera Rogiers, Annalaura Stammati, Maciej Stepnick, Hanna Tahti, Augusto Vitale, eplacement

Thursday February 26

13.00-14.00	**Registration**
14.00-14.30	**Welcome** Alessandro Di Domenico and Filippo Belardelli Annalaura Stammati, Franca Fassio **START-UP Project** Vera Rogiers, Chair of Ecopa
14.30-15.00	**INTRODUCTION** **Chairperson:** Maciej Stepnick and Kirsi Vahakangas · **Conclusions on refinement from Animalsee EU Project:** Flavia Zucco · **Refinement opportunities - an overview:** Peter Thornton · **Point of view of a philosopher:** Simone Pollo
15.00-15.10	**Discussion**
15.10-15.40	**ROUND TABLE 1: Housing and legal aspects** **Chairperson:** Enrico Alleva and Hanna Tahti · **Refinement in 609:** Kai Pelkonen · **Environmental enrichment and standardization:** Simone Macrì · **Focus on Refinement in a Pharmaceutical company is good for business:** Jan L.Ottesen
15.40-16.20	**Discussion**
16.20-16.40	**Coffee break**

16.40-17.35	**ROUND TABLE 2: Recent methodologies in refinement** **Chairperson:** Patrizia Costa and Peter Maier · **New imaging techniques:** Vicky Cavéliers · **Positive training in non-human primates (including showing of a DVD):** Fanélie Wanert · **Stress reduction in working with Beagle dogs; a win-win situation:** Pieter Verbost · **Animal research in a global pharmaceutical and chemical company:** Pierre Coërs
17.35-18.15	**Discussion**

Friday February 27

09.00-09.40	**ROUND TABLE 3: Animal models in human diseases** **Chairpersons:** Christoph Wiessner and Germano Oberto (Farmindustria representative) · **Refinement in non-human primate studies of Parkinson disease:** Augusto Vitale · **Application of Refinement in transgenic mice:** Igor Branchi · **Refinement in Multiple Sclerosis (MS) studies:** Valeria Muzio · **Refinement in animal epilepsy models:** Ralph Clinckers
09.40-10.20	**Discussion**
10.20-10.50	**Coffee break**
10.50-11.20	**ROUND TABLE 4: Refinement in drug development process** **Chairperson:** Bernward Garthoff and Josè Castell · **Refinement in the ADME studies:** Olavi Pelkonen · **EDQM activities for refinement of animal experiments in the field of quality control of vaccines:** Karl-Heinz Buchheit · **Refinement in the development of biopharmaceuticals:** Gisbert Sponer
11.20-12.00	**Discussion**
12.00-13.00	**Final discussion and conclusions: the Chairpersons**

Note: written questions will be collected during the workshop and discussed in the final discussion


Speakers and Chairpersons

Enrico Alleva, Cell Biology an Neuroscience Department, ISS, Rome, Italy
Igor Branchi, Cell Biology an Neuroscience Department, ISS, Rome, Italy
Karl-Heinz Buchheit, Eu. Directorate Quality of Medicines & HealthCare (EDQM), Strasbourg, France
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Germano Oberto, Scientific Director Research Toxicology Centre (RTC), Pomezia, Italy
Jan Lund Ottesen, Animal Unit , Novo Nordisk A/S, Denmark
Kai Pelkonen, Ministry of Agriculture and Forestry, Finland
Olavi Pelkonen, Department of Pharmacology and Toxicology, University of Oulu, Finland
Simone Pollo, Philosophical Studies Departement, University La Sapienza, Rome, Italy
Vera Rogiers, Vrije Universiteit Bruxelles, Belgium, Chair of Ecopa
Gisbert Sponer, Bioassay GmbH, Gerlingen, Germany
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Maciej Stepnick, Nofer Institute of Occupational Medicine, Chair of POLCOPA, Lodz, Poland
Hanna Tahti, FICAM, Medical School, University of Tampere, Finland
Peter Thornton, ASPI, Home Office, London, UK
Kirsi Vahakangas, Chair of Fincopa, Dept. Pharmacol. and Toxicol., University of Kuopio, Finland
Pieter Verbost, Toxicology & Drug Disposition Deputy Department, Schering-Plough,
Augusto Vitale, Cell Biology and Neuroscience Department , ISS, Rome, Italy
Fanèlie Wanert, Centre de Primatologie de l'Université de Strasbourg, France
Christoph Wiessner, Merck Serono, Genève, Switzerland
Flavia Zucco, Institute of Neurobiology and Molecular Medicine, CNR, Rome, Italy

Invited Participants

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Rosalia Bertorelli, Schering Plough Research Inst., San Raffaele Science Park (Farmindustria), Milano,Italy
Roberta Bonafè, Centro Ricerche Bracco (Farmindustria), Colleretto Giacosa, Italy
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Rosangela Ferretti, Sigma-Tau, Pomezia, Italy
Paolo Galloni, Enea Casaccia, Rome, Italy
Gerhard Gstraunthaler, Innsbruck Medical University, Austria
Tuula Heinonen, FICAM, Medical School, University of Tampere, Finland
Tarja Kohila, Laboratory of Experimental Animals, Helsinki University, Finland
Michela Kuan, LAV (antivivisection League), Rome, Italy
Lucia Luperi, RTC (Farmindustria), Pomezia, Italy
Mario Marubini, Merck Serono (Farmindustria), Ivrea, Italy
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Barbara Musi, Gambro R&D, Lund, Sweden
Marianna Norring, Juliana von Wendt'Foundation, Finland
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Markku Pasanen, Department of Pharmacology and Toxicology, University of Kuopio, Finland
Walter Pfaller, Innsbruck Medical University, Austria
Joanna Piasecka-Zelga, Nofer Institute of Occupational Medicine, Lodz, Poland
Marta Piscitelli, Enea Casaccia, Rome, Italy
Marcello Raspa, CNR, Roma, Italy
Carla Rocchi, National Body for Animal Prevention, Rome, Italy
Lasse Saloranta, Orion Pharma, Turku, Finland
Andrea Tamellini, GlaxoSmithKline (Farmindustria), Verona, Italy
Emanuela Testai, Environment and Primary Prevention Department, ISS, Roma, Italy
Jan-Willem van der Laan, RIVM, The Netherlands

The workshop is organized within the frame of the EU-sponsored project "Scientific and technological issues in 3R alternatives research in the process of drug development and Union politics" (acronym: START-UP) addressing the Work Program topic: HEALTH-2007-1.3-2. The basic aim of the START-UP project is to identify bottlenecks in the implementation of refinement, reduction and replacement of animal experimentation in drug discovery and development. This will help in identifying the best *in vitro* and *in vivo* systems, and speed up the whole process of drug development. The present obstacles on scientific, technological, as well as on political and environmental levels (including regulatory issues), are rate-limiting steps in developing new pharmaceuticals, whether defined chemical substances or biological entities.

Technical Secretariat

Laura Turco, ISS
Franco Zampaglioni, ISS

3.2 Transition to a toxicity pathway-based paradigm

More and more scientists and governments agree on the need for a new paradigm for safety assessment testing, based on the understanding of toxicity pathways at cellular and genomic levels. The United States National Research Council (NRC) commissioned by the U.S. Environmental Protection Agency (EPA) in 2007 developed an approach for toxicity testing focused on toxicity pathways, later published under the title of Toxicity Testing in the 21st Century: a Vision and a Strategy.

AXLR8 will be the first FP7 project to focus on 'toxicity pathways'. This Coordination Action will bring together European and international researchers, promoting the discussion and the development of the so-called 21st century paradigm in toxicology.

At the moment this book is being prepared, AXLR8 is being negotiated. It is still expected to start in 2009.

AXLR8

Accelerating the transition to a toxicity pathway-based paradigm for chemical safety assessment through internationally co-ordinated research and technology development

Proposal number:	241958
Project type:	Coordination Action (FP7)
EC contribution:	€ 555 224
Starting date:	2009 (date to be confirmed)
Duration:	40 months

Background

Current reliance on high-dose animal toxicity studies and the application of default extrapolation procedures is a source of considerable uncertainty in human-health risk assessments. Additionally, conventional animal tests are in general quite time consuming, low throughput, costly in both economic and animal welfare terms, and offer little mechanistic understanding of how chemicals act in the body. However, dramatic advancements in molecular and cellular biology in recent years have made available a wide range of new tools – including functional genomics, proteomics, metabolomics, high data content screening, and systems biology – for studying the effects of chemical stressors on cells, tissues and organisms in a rapid and cost-efficient manner. This convergence of factors, coupled with the need to evaluate the safety of an increasingly large number of chemicals and their mixtures, has prompted some of the world's leading scientific authorities to call for a fundamental paradigm shift in toxicology.

Instead of focusing on signs of gross toxicity at high doses in living animals, an alternative approach advocated by the US National Research Council and others is to work towards a mechanistic understanding of how chemicals interact with cellular response pathways in the human body at environmentally relevant exposure levels. As critical pathways are identified, human cell-based assays can be developed to study chemical interactions at key cellular and molecular targets within a pathway, and through robotic automation, cell-based *in vitro* methods can enable the high throughput testing of thousands of substances in a single day. Data from toxicity pathway assays could then be integrated and interpreted with the aid of systems biology tools of the cellular 'circuitry' controlling pathway function, together with pharmacokinetic modelling to relate *in vitro* conditions to expected real-world human exposure levels.

The technology required to move toward a '21st century' paradigm in toxicology is already available or in a state of advanced development. What is needed is the elucidation of unmapped toxicity pathways, linking them closely with relevant human health outcomes, the development of appropriate high throughput cellular assays, improved bioinformatics to analyse large databases, and to combine this information through systems biology. In addition, pharmacokinetic models must be established to describe the concentrations of chemicals over time. Taken together, this should enable the prediction of dose-response *in vitro*, and the effective integration of these approaches for risk assessment purposes. This new paradigm will change the traditional toxicity testing and risk assessment approaches and needs reflection and acceptance not only by the scientific community but also by the all stakeholders who are involved in health and safety issues.

Objectives

AXLR8 is a multi-year coordination action designed to fulfil the growing need for a focal point for discussion and coordination among key European and international research, development and translational activities related to the implementation of a 21st century paradigm in toxicology. This project will provide a wide range of tools and opportunities for increased networking, information exchange, problem solving, strategic planning and collaboration among relevant scientific disciplines and stakeholder groups, both within the EU and globally. AXLR8 also aims to accelerate the acceptance and use of suitable animal replacement, reduction and refinement methods into regulatory decision-making, and to pave the way for a transition to 21st century technologies designed to detect biologically significant perturbations of toxicity pathways.

Approach

A cornerstone of the AXLR8 project will be the establishment of a pan-European scientific dialogue and advisory committee comprising leading experts from the academic, industry, government and regulatory sectors, and relevant EU- and Member State-funded initiatives as well as key international experts, to promote enhanced interdisciplinary and global communication, coordination, collaboration and exchange of best practices.

AXLR8 will organise three expert workshops to map existing research results germane to a pathway-based paradigm in toxicology and identify needs and priorities for future R&D and targeted funding. These workshops will invite participation from key sectoral experts from relevant disciplines, and thereby promote focused dialogue and the creation of new synergies among these communities. A public report will be prepared following each workshop, as well as one or more manuscripts for submission to a peer reviewed journal.

Promotion of greater scientific, stakeholder and public awareness and communication regarding 21st century approaches to toxicology and risk assessment will be fostered through the creation of a specific online 'community of practice' discussion forum on http://alttox.org/. Additionally, proactive outreach will be undertaken via presentations at several key EU toxicological conferences each year.

AXLR8 will convene an additional expert meeting to identify bottlenecks and barriers to the acceptance and use of 3R methods and testing strategies to their full potential for regulatory purposes. On the basis of these findings and expert advice regarding strategies for overcoming barriers, AXLR8 will work with regulatory authorities to implement new procedures, as needed, to facilitate a more expeditious and efficient uptake of both existing and second-generation alternative approaches to reduce reliance on *in vivo* testing.

Finally, AXLR8 will produce an authoritative report of the state of the science, including a practical road map detailing priority research and funding targets, in order to ensure a prominent role for EU scientists in this rapidly developing global research area.

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3.3 A possible strategy for the replacement of animals in repeated dose systemic toxicity testing

In DG RTD, several FP6 projects dealing with *in vitro* and *in silico* methods for human safety assessment are still running and the expectations for their final results are high. FP7 started a significant number of new initiatives ranging from integrated testing strategies to coordination and support actions aimed at optimising the use of the limited financial resources.

None of the supported projects, however, addresses the development of alternatives to *in vivo* repeated dose systemic toxicity testing, which consumes the largest number of laboratory animals in risk assessment, and for which no alternatives are currently available.

The approach designed in early 2009 by a group of experts invited by DG RTD and The European Cosmetics Association (COLIPA), foresees a long-term research task to achieve the full replacement of animal tests in safety assessment. This exercise put emphasis on the safety assessment of potential ingredients of cosmetic products.

The proposed research plan of the first five-year period would include the following building blocks:

(i) the development and use of advanced organ-simulating devices as alternatives for long-term toxicity testing;

(ii) the optimisation of current methodologies and development of novel methods to achieve functional differentiation of human-based target cells *in vitro* that change the focus from rodent systems to the more refined human models, allowing identification of human toxicological biomarkers and endpoints;

(iii) the establishment of endpoints and intermediate markers in human-based target cells with relevance for repeated dose systemic toxicity testing;

(iv) the optimisation of computational modelling and estimation techniques;

(v) the exploitation of systems biology, physiologically based pharmacokinetic (PBPK) modelling and (Q)SAR approaches, for the development of predictive causal computer models to forecast the toxicological potential of previously uncharacterised chemical compounds.

Additionally, this research strategy would strongly benefit from the establishment of a dedicated Web-based 'warehouse', a database and a depository of selected model compounds, a bank for the cells, cell lines and tissues of relevance for *in vitro* toxicity testing (Figure 1).

The compilation of information and research data by a centralised 'data warehouse' would allow for an integrated data analysis with the advantage that the outcome would be fed back into the ongoing research in order to further steer experiments accordingly, allowing improvement in an iterative way.

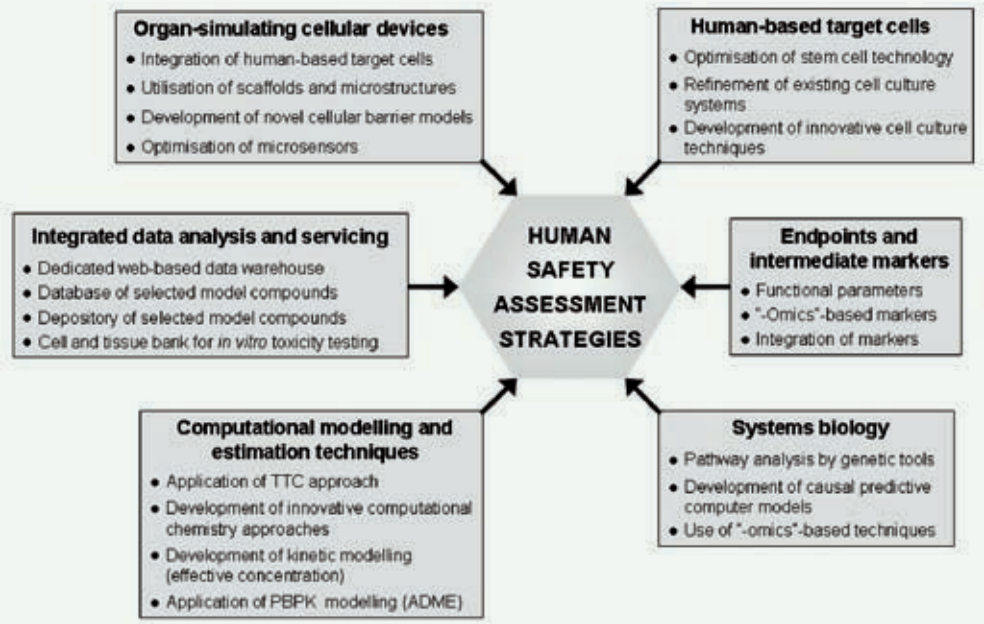

Figure 1. Building blocks of a possible strategy for the development of alternatives to repeated dose systemic toxicity testing of cosmetic ingredients.

The scientific community engaged on 3Rs research is also lacking a database of selected model compounds, meeting the highest quality standards. Chemicals in such a database should be backed up by high-quality repeated-dose toxicity *in vivo* data from animal studies and, whenever available, experience in humans. The database should cover cosmetic ingredients, industrial chemicals, pharmaceuticals, plant protection products and biocides that meet the same high-quality criteria. In addition, a list of selected model compounds, standard operating procedures for data quality control, processing and analyses should be provided. Whenever compounds are needed for training or validation purposes, these should be selected from this toxicological database in correspondence with their targeted mode of action in chronic toxicity.

Moreover, it seems crucial that such concerted research effort should be supported by the establishment of a bank of cells, cell lines and tissues, as well as a depository of selected chemicals, sometimes a limitation to research in the past, for common use within the network.

Within each building block, the experts highlighted a number of important sub-topics as shown in Figure 1.

These sub-topics are described in detail in the text below.

(i) Exploitation of organ-simulating cellular devices as alternatives for long-term toxicity testing

1) Integration of target and metabolising cells to simulate multi-organ-related toxicity in vitro

One potential approach to replace or at least reduce animal experimentation in repeated dose systemic toxicity testing is the use of organ-simulating devices. These may be based directly on complex three-dimensional architectures of cultured human-based target cells in a bioreactor designed to reproduce *in vivo*-like functional activity over extended periods.

Bioreactors need to be designed to support both assembly and homeostasis of a complex architecture within an affordable framework. The system must include facilities for drug application and for read-out of the assay. A correlated requirement is for appropriate human cells preferably differentiated *in situ* to represent a particular phenotype. More detailed understanding and control of the chemical composition of the culture media is required in order to maintain all parameters within a specific range of action. Miniaturisation should be employed both to emulate substructures in human organs and to facilitate high throughput test designs.

An alternative approach is the use of organ-simulating devices based on robust monocultures or supracellular structures designed to mimic the essential molecular interactions in a biochemical simulator that can feed information to an *in silico* model.

Despite rapid advances in the individual technical fields required to assemble such devices, predictive procedures for assessing human exposure based on such technologies remain rudimentary and restricted to the research level. A concerted interdisciplinary effort is required to elevate these methodologies to a level suitable for practical application.

2) Utilisation of scaffolds and microstructures to optimise the cellular microenvironment

An alternative approach to organ-simulating devices is to reduce the complexity of the bio-assembly in the device by substituting this with artificial microstructures and scaffolds. Two- or three-dimensional arrays of monocultures connected chemically using

fluidics could deliver more robust systems designed to mimic functionality rather than to mimic morphology. Nanostructured materials, synthetic and natural polymer technology, or self-assembled systems may provide the basis for the three-dimensional structures required to support cell proliferation while oxygen and nutrient supply are delivered using artificial microsystems or fluidic transfers. Likewise, the metabolic interactions may be effected by engineered microfluidic connections rather than depending on wholly natural assemblies to deliver complex structural interactions. Research is needed into appropriate structures to support sustainable cell growth in an artificial environment where the demands of biocompatibility are ameliorated compared to the restrictions placed on implantable systems. These then need to be integrated into functional systems designed using novel methods for the construction of three-dimensional fluidic architectures to produce an integrated system with the potential to meet industrial needs for an inexpensive test facility. It is thus necessary to develop stable cell culture systems that are supported by scaffolds and microstructures that mimic the natural microenvironment.

3) *Development of novel cellular barrier models relevant to systemic exposure*

For the assessment of topical applications, a number of cellular barrier models are currently available, being used as well in research as for commercial purposes. No comparable system, however, seems to be available for systemic exposure. *In vitro* models are required for detecting damage to various cellular barriers such as the renal epithelium, the intestinal barrier and the blood-brain barrier, after acute and chronic exposure to chemicals and products of various kinds. Measurement of transport or leakage of indicator compounds, direct non-invasive monitoring of the intact tissue or measurement of indirect effects correlating to barrier effects, could provide viable routes forward. Appropriate culture techniques coupled with advanced sensing technologies are required to furnish a cost-effective solution. Therefore, appropriate cellular barrier models for systemic exposure to measure the bioavailability of chemicals in the target cells is required.

4) *Optimisation of microsensors to monitor tissue responses in organ-simulating devices*

Bioreactors need to be designed to support homeostasis of complex tissue architectures by incorporating appropriate ways to monitor the response of the system, using non-invasive sensors, in-built microsensors or molecular signalling, for example. Work is required to create sensing strategies or structures that can probe the three-dimensional complexity of cultured material with sufficient resolution and accuracy to support both homeostasis and/or the acquisition of transitional changes that can be more rapidly correlated to toxic responses than can be achieved using conventional outcomes. This output could usefully be multifactorial in order to furnish richer information about the status and response of the artificial organ. A further progression would be to reduce the organ-simulating device to representative arrays of supramolecular structures designed to mimic selected elements of cell susceptibility and to supplement this chemical information with computational modelling. This latter approach may be considered as a hybrid, which supplements computer modelling with new real-time data on actual biochemical interactions generated by the target compound. It is thus essential to develop detection tools for non-invasive monitoring of functional behaviour of cells, whether treated with chemicals or not.

(ii) Optimisation of current methodologies and development of innovative strategies to control functional differentiation of human-based cells *in vitro*

1) Optimisation of stem cell technology as a source for human-based target cells for toxicological purposes

Stem cells, either of embryonic or adult origin, foresee a virtually unlimited source of different cell types. In recent years, many efforts have been focused on the *in vitro* differentiation of stem cells into cells that are of toxicological relevance, e.g. hepatic cells. There are, however, still a number of hurdles to be overcome. These include the difficulty of reproducibly generating organ-specific cells, as well as the challenge of scaling up the production of such cells in the quantities required for high throughput analysis.

Current methods for generating early somatic tissue lineages from stem cells can efficiently yield progenitor cell types expressing biomarkers that are shared with their *in vivo* counterparts. However, efficiency declines as these progenitor populations are driven further towards mature differentiated cells. Therefore, improvements will be needed, especially at intermediate stages of differentiation between stem cell and mature cell states, to increase final yields of mature, organ-specific cells. Likewise, marked improvements are needed for maintaining the *in vitro* proliferative status in such intermediate cell types (transit-amplifying cells) as a means of obtaining sufficient cells for large-scale analyses. In particular, the recently introduced strategy to achieve differentiated (organ-specific) cells by sequential exposure of stem cells to a series of growth factors, reflecting (*in vivo*) embryogenesis, seems promising and deserves further attention. Stability of the differentiated state and maintenance of a relevant phenotype, i.e. for toxicity testing, are clearly necessary for successful delivery of both target and metabolically active cell types.

2) Refinement of cell culture systems for long-term toxicity testing

The maintenance of the differentiated phenotype of cells in culture, both at the morphological level and at the functional level, is a key element for the development of cell culture systems that can be used for long-term toxicity testing. Current strategies to favour cellular differentiation or to counteract cellular dedifferentiation *in vitro* are mainly based on mimicking the physiological *in vivo* situation. Typical examples, especially for human primary cells and cell lines, include culture on extracellular matrix proteins (restoration of cell-extracellular matrix contacts), co-cultivation with another cell type (re-introduction of cell-cell interactions), and addition of physiologically relevant chemical compounds to the cell culture medium. In addition, tissue slices offer the advantage that they keep the original cellular architecture, but they also suffer from dedifferentiation and can thus only be used for short-term purposes. The research outcome needed in this area is thus the development of cell culture systems in which the differentiated phenotype, in particular the toxicologically relevant functionality of the target organ, can be maintained for at least two weeks.

3) Exploitation of emerging mechanistically driven methods controlling cellular differentiation

Basic research to clarify the molecular mechanisms that drive cellular (de) differentiation is required, which in turn could open new perspectives for the development of innovative methods to induce and/or maintain differentiation of cells in culture. For instance,

interfering with posttranslational protein modifications may substantially enhance functional differentiation over the long term. Genetic and epigenetic approaches, including the introduction of differentiation-promoting genes into the genome and the modification of the chromatin structure in favour of differentiation-promoting transcriptional activity respectively, allow the direction of the differentiation process at the most upstream regulatory level. It is evident that '-omics' technology in all its aspects plays a key role in these new strategies. Another major challenge lies in altering the expression profile of microRNA species, which are essential determinants of cellular differentiation. Such innovative 'cellular differentiation therapies' are still in their infancy, and need to be further exploited before they can be optimally used in (long-term) *in vitro* modelling.

(iii) Establishment of endpoints and intermediate markers in human-based target cells with relevance for repeated dose systemic toxicity testing

In order to enhance the predictive capacity of the screening systems the development of a test battery integrating '-omics'-based and functional parameters is needed.

Research should be carried out on covering aspects of target cells, and the determination and evaluation of genomic, proteomic, metabonomic and system biological markers with proved predictive capacity and strong relevance for human toxicity. Functional endpoints, as the most relevant parameters of the physiological *in vivo* situation of the organism, are highly relevant to assess the toxicological effects and should be regarded as refinement of the '-omics'-based markers.

(iv) Computational modelling and estimation techniques

1) Threshold of toxicological concern approach for the safety assessment of cosmetic ingredients

The threshold of toxicological concern (TTC) concept is based upon the evidence that potent toxic chemicals would not be expected to cause harm to humans if the exposure is below a defined threshold. Separate evaluations by industry and by independent experts at European level came to the conclusion that improvement and adaptation of the concept seems necessary before it could be applied to cosmetic ingredients. Further efforts in this direction should be focused on the development and validation of toxicological (e.g. carcinogenicity) databases, since the actually existing carcinogenicity database is 25 years old and was expanded about 10 years ago only to a limited extent. New databases should be established, e.g. for carcinogenicity, based on substances classified as human carcinogens, probably or likely human carcinogens, and for non-cancer toxicological endpoints the focus should be in particular on the inclusion of recent toxicity data. The following aspects with regard to data entry in the toxicological databases need to be addressed: (*i*) presence of up-to-date and peer-reviewed data; (*ii*) display of data under the same focus as existing data; (*iii*) consideration of correction factors (e.g. allometry, study duration, study outcome); and (*iv*) inclusion of sufficient structure analogues to cosmetic ingredients.

2) Innovative computational chemistry approaches in the safety assessment of cosmetic ingredients

Existing approaches such as (Q)SAR and read-across methods need to be refined and overall strategies devised incorporating kinetic and metabolic studies to permit quantitative

interpretation of results in terms of consumer risk. If *in silico* approaches are used routinely in the cosmetics industry, it will be important to identify reliable packages that can be supported over the long term. Grouping approaches, known now for the evaluation of flavourings for example, could potentially be considered for ingredients of cosmetics. (Q)SAR and read-across approaches for the purpose of long-term toxicity prediction of cosmetic ingredients should be optimised.

3) Predicting the dose at the target level upon long-term exposure

Tools for kinetic modelling should be developed to predict target organ concentrations and the accumulation of chemicals and their metabolites in the context of exposure. Actual concentrations should take into account potential variables such as binding to proteins and specific components of *in vitro* systems, e.g. plastics of the culture dish, three-dimensional networks and scaffolds.

4) PBPK modelling in the safety assessment of cosmetic ingredients

PBPK modelling should be applied to integrate *in vitro* and *in silico* data in order to develop ADME (complete absorption, distribution, metabolism and excretion) models for decision making and risk assessment. This approach represents a way of integrating physicochemical, *in vitro*, human and animal data and computational methods in order to develop a complete model of ADME for a particular compound. No observable (adverse) effect concentration (NO(A)EC) as a measure of toxicity should be derived from *in vitro* repeated dose systems and PBPK should allow the prediction of a corresponding *in vivo* concentration dose. One may also integrate other parameters, e.g. an improved exposure assessment using markers to support the application of the TTC.

(v) Systems biology for the development of predictive causal computer models

1) Identification and analysis of pathways relevant to long-term toxicity by genetic tools

One of the goals of this research strategy is to develop a battery of genetic tools more selective than current animal testing procedures, and thus more predictive for long-term toxicity. These genetic tools should be 'across species' in order to comprehensively cover the possible target and response pathways of the molecules, they should be highly reproducible and selective (inbred strains, deletion strains) and should be easily accessible for the '-omics'-based technologies such as those described in Section **(v) 2)**. Such tools should include relatively simple systems such as yeast, cells/cell lines of animal or human origin and well-characterised human cell lines that are much closer to the human pharmacokinetic profile. Experimental designs should include a panel of selected compounds and the challenge of the different systems with different dosages of these compounds. The outcome of this multi-species analysis should be the identification of conserved (and non-conserved) target pathways along with the long-term toxicity endpoints for the compounds (and compound classes) under analysis.

2) Use of '-omics'- based techniques to identify mechanistic pathways involved in long-term toxicity effects

Research is needed on the measurement of changes upon compound treatment on a global scale with respect to essential biological activities (e.g. transcriptome, epigenome, proteome, metabolome) in different model systems in order to identify mechanistic pathways involved in long-term toxicity effects. The '-omics'-based technology should be based on cutting-edge technology such as next-generation sequencing (RNA-

Seq, MEDIP-Seq), quantitative proteomics and NMR/MS technology. Additionally, experimental validation technologies such as RNAi screens, ChIP-Seq and protein over- and under-expression of selected compound targets should be applied. The experimental work should be accompanied by the development and application of data integration tools and high-performance computational analysis methods for the different technologies. The outcome of the genome-wide and validation screens should be the identification of response pathways for the long-term toxicity effects of the different compounds.

3) *Development of causal predictive computer models for long-term toxicity effects*

Modelling systems is another possible approach to the development of a tool to predict the response to repeated dose systemic toxicity of chemical compounds. Research on this subject should address specific tissues, specific developmental stages, or ultimately the entire organism. Model systems should be able to integrate experimental data on different levels of cellular information and of the genetic systems. Modelling methodology can include quantitative and qualitative methods. The approaches should be highly automated and should be able to access large collections of network information. Models are typically large scale, so the modelling strategy should incorporate computational solutions for performing computing intensive simulations, for example grid computing.

4. ALPHABETICAL INDEX OF PROJECTS

5. INDEX OF COORDINATORS

European Commission

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Alternative Testing Strategies

Replacing the need for animal experiments, reducing the number of animals required and refining the procedures to improve animal welfare when the use of animals is unavoidable, also known as the 3Rs Principle, are the underlying concepts for the development of alternative testing strategies used in pharmaceutical discovery and development, and in safety assessment of chemicals. Research into alternative testing strategies is part of the EU's Sixth and Seventh RTD Framework Programmes (FP6 and FP7: 2002-2013). The purpose of this publication is to present past and ongoing activities in this field of FP6 and FP7 and the results generated. A particular focus of the work is on the development of *in vitro* methods and on methods that can be validated to meet international standards, achieve approval of regulatory authorities and be adopted by industry.

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