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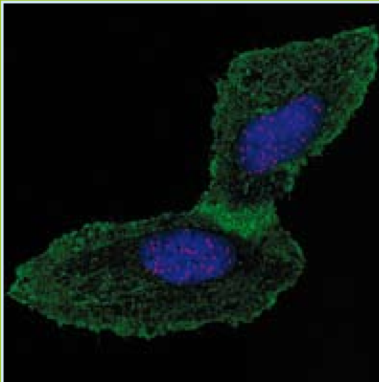

Alternative Testing Strategies

Replacing, reducing and refining use of animals in research

PROJECT SYNOPSES

SIXTH FRAMEWORK PROGRAMME

EU-SUPPORTED RESEARCH 2002-2006

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EUROPEAN COMMISSION

ALTERNATIVE TESTING STRATEGIES

Replacing, reducing and refining use of animals in research

Edited by
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Directorate-General for Research

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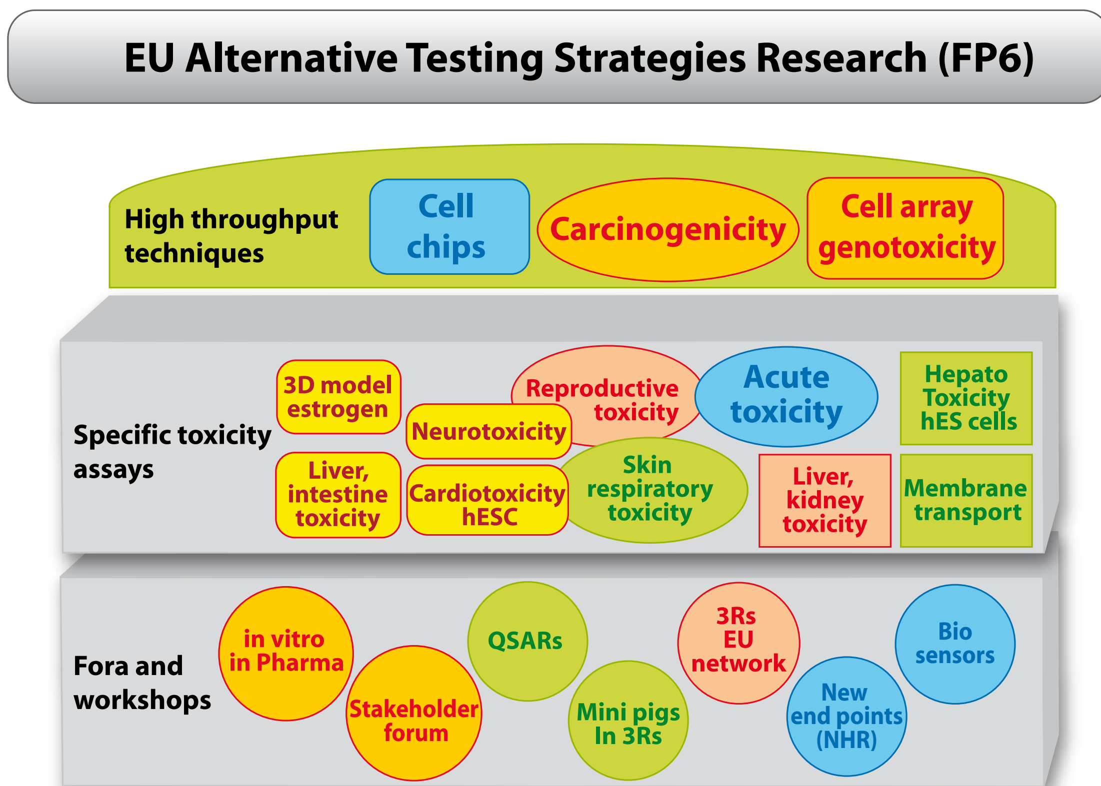
INTRODUCTION

'Alternative testing strategies' refers to efforts to replace animals in experimentation as part of the "Three Rs" approach of replacement, reduction and refinement. Developing robust and effective *in vitro* tests has been a priority for EU research programmes since the 1980s. The challenge is to develop alternative methods which can be validated according to international standards, be recognised by regulatory authorities and be adopted by industry. A particular application of the methods now is the EU's registration, evaluation and authorisation of chemicals policy (REACH).

The purpose of this compilation of information on EU-supported research in alternative testing strategies is to demonstrate the range of activities undertaken and initial results obtained in this field during the course of the EU's Sixth Framework Programme for Research (2002-2006), notably under the heading 'Applications of knowledge and technologies in the field of genomics and biotechnology for health' in the 'Life sciences, genomics and biotechnology for health' thematic priority. Information is also provided on projects being supported following the first call for proposals made by the Health priority of the newly-launched Seventh Framework Programme (2007-2013); this indicates that research efforts towards alternative testing are continuing, and further support to the area is envisaged as the programme develops.

The projects described fall into three groups: high-throughput techniques, specific toxicity assays and projects focusing on specific scientific, social or ethical aspects and taking the form of fora and workshops. The projects are placed in

alphabetical order in the book and a summary of the main topics covered by the activities is shown in schematic version in the figure below:



▲ Main topics of alternative testing strategies research projects described in this review. Different colours refer to different calls for proposals. Shapes: oval – integrated projects; rectangle – specific targeted research project; circle – specific support action.

The aim of the projects described here was to create technological platforms for the development of alternative testing strategies. To achieve this, projects were built around five different funding schemes:

Integrated projects (IP). These are the largest size projects and integrate a range of different activities, such as research, demonstration and training. They also permit projects to take a multi-disciplinary approach, to link underlying biology and tool development and enable scientists, clinicians and other stakeholders to work together to achieve their deliverables.

Specific Targeted Research Projects (STREP). These are smaller projects which focus on specific research issues. They may have an applied focus but are less multi-disciplinary and wide-ranging than the Integrated Projects.

SME-Specific Targeted Research Projects. Targeted Research Projects designed to encourage research and innovation efforts of small and

medium-sized enterprises (SMEs) and where research-led SMEs play a leading role.

Specific Support Actions (SSA) for training, conferences or prospective studies in support of the programme.

European research projects are encouraged to be open towards the general public and to engage with stakeholders and interest groups. This is particularly important in the field of alternative testing which is of considerable public interest. Accordingly many projects organise public meetings and dialogue and set up websites on the consortium, the research and results.

Website addresses are given in the details of each project and provide more up-to-date information than this publication.

The number of projects supported by the different funding schemes and the EC financial contribution to them is shown in the table below. It can be seen that during the Sixth FP overall 21 projects were supported with an EC contribution of over EUR 63 million; this was distributed among about 255 research teams, almost 55 of which are SMEs. In the Seventh FP, five projects are under contract negotiation and envisage an EC contribution of around EUR 30 million.

Funding scheme	Number of projects	EC financial contribution (million €)
Sixth FP		
Integrated project	4	39.5
Specific Targeted Research Project	4	8.7
SME-Specific Targeted Research Project	5	13.0
Specific Support Action	8	2.1
Total	21	63.3
Seventh FP (First Call)	5	30.4 (planned)

▲ Numbers of projects supported and EC financial contribution to alternative testing strategies research

It is clear from this compilation that alternative testing strategies is an extremely active area of research for European scientists both from the public and private sectors. These efforts in the health programme are complemented by related research undertaken by the nanosciences, nanotechnologies, materials & new production technologies programme and by the environment programme of the Seventh FP.

INTRODUCTION

While the desire to avoid the use of animals in safety testing is straightforward and easily expressed, making this a reality is complex. The projects described in this book concern the first step in the process, namely the development of new tools, techniques and methods that can be used in safety testing in pharmaceutical, chemical, cosmetic or other industries. This step is essentially methodological, is a key one and is one dependent on scientific innovation. However to get these new methods adopted, they need to be scientifically validated by comparison with existing standard methods. This task is undertaken by the European Centre for the Validation of Alternative Methods (http://ecvam.jrc.it), which is a partner in some of the projects. Even once validated, there are still hurdles to be overcome for the new tests to become standard practice. For instance, different countries have different regulatory requirements and industry has to invest in setting up the new methods. This step is facilitated through the European Partnership for Alternative Approaches to Animal Testing (http://ec.europa.eu/enterprise/epaa/index_en.htm), which is a joint initiative from the European Commission and a number of companies and trade federations active in various industrial sectors whose purpose is to promote the development of new "Three Rs" methods as modern alternative approaches to safety testing. Authorities in charge of implementation and enforcement have a particular role in ensuring that validated new techniques are used.

In conclusion, it can be seen that replacement of animals in safety testing involves many steps and different sectors, and that nothing would be possible in this field without scientific innovation.

Alternative Testing Strategies
Genomics & Biotechnology for Health

ACUTETOX

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

Contract No	**LSHB-CT-2004-512051**
Project type	**Integrated Project**
EC contribution	**€ 9 000 000**
Starting date	**1 January 2005**
Duration	**60 months**
Website	**www.acutetox.org**

Background and 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 9 scientific Work Packages (WPs):

- WP1: The generation of a high quality *in vivo* database;
- WP2: The generation of a high quality *in vitro* database;
- WP3: Iterative amendment of the testing strategy;
- WP4: New cell systems and new end-points;
- WP5: Alerts and correctors in toxicity screening (I): Role of ADE;
- WP6: Alerts and correctors in toxicity screening (II): Role of metabolism;
- WP7: Alerts and correctors in toxicity screening (III): Role of target organ toxicity;
- WP8: Technical optimisation of the amended test strategy;
- WP9: 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 (WP1 and WP2);
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 (WPs 5, 6, 7.1, 7.2, 7.3);
3. Exploration of innovative tools and cellular systems to identify new end-points and strategies to better anticipate animal and human toxicity (WP4);
4. Design of a simple, robust and reliable *in vitro* test strategy amenable for robotic testing, associated with the prediction model for acute toxicity (WPs 3, 8, and 9).

ACUTETOX

Approach and methodology:

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 selected mainly from previous studies (MEIC and ECVAM/ICCVAM validation, for example) are being tested in different *in vitro* and *in silico* assays. 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 two years.

Expected outcome:

The formal validation of the definitive 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.

Main findings:

The work in WP1 and WP2 has been finalised. In WP1, animal and human data for the 97 ACuteTox reference compounds were compiled. The database contains LD50 values from 2 206 animal studies, as well as human data from 2 902 case reports, including acute sub-lethal and lethal blood concentration data. Descriptive summaries containing physico-chemical data, LD50 values, human toxicity data, pharmacokinetics-/toxicokinetics- data, metabolism, toxicological mechanisms, and target organs for all 97 reference chemicals have been recorded.

The testing of the ACuteTox reference chemicals in six basal cytotoxicity tests (WP2) has been completed. Data for most of the 97 reference compounds are available for the Fa32/NR uptake, Fa32/protein content, 3T3/NR uptake and NHK/NR uptake systems. Data are available for half of the compounds in the HepG2/protein content and HL60/ATP content systems. Data from WP1 and WP2, as well as from WP4 to WP7, are stored in AcuBase, a database developed in WP3 to facilitate Standard Operating Procedures (SOPs) storage, data transfer from all partners and statistical analysis of larger data sets.

The data from WP1 and WP2 have been compared in some preliminary multivariate analyses with the aim of identifying additional outliers that will be tested by the Partners before the final selection of methods that will enter the prevalidation phase. One of the aims of the project is to adapt the methods of the testing strategy to high-throughput screening (HTS). In WP3, a number of test assays such as the 3T3/NR uptake and HepG2/MTT assays have successfully been adapted to two commercially available HTS robotic platforms.

In WP4 through WP7, the testing of the first 2 sets of reference chemicals (no. 1-46) has continued and the results are reported in AcuBase. This testing will continue until month 30 when the data sets will be integrated in the *in vitro*/*in vivo* comparisons with the aim of selecting methods for improving the correlation and thus becoming candidates for inclusion in the testing strategy.

The aim of WP4 is to provide an alternative way for improving the prediction of acute toxicity by incorporating 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 as well as effects on the CFU-GM and progenitors of megakaryocytes have been measured for 20 chemicals and the results are

now being analysed. The miniaturising of the Cytomic Panels for Cytotoxicity and Oxidative Stress Screening, as well as the testing of 20 reference chemicals, have been finalised and the results show good correlation with human blood LC50 values.

The most crucial parts of the kinetic behaviour have been studied in WP5. For this purpose, the determination of kinetic parameters is being performed either by experimental, *in vitro* tests or computer-based kinetic modelling. Neural network methodologies that are useful for estimating oral absorption and blood brain barrier passage have been developed. Results from three variants of the Caco-2 model for prediction of oral absorption, used in three different laboratories, have been compared. So far, the results show good agreement between the different variants. Toxicity studies and permeability studies using *in vitro* BBB models have been performed for 22 and 19 compounds respectively, showing relatively good correlation with *in vivo* data. Another aim of WP5 is 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 microextraction. This technique was proven to accurately measure the free concentration of compounds such as PAHs, being easy to use. Finally, *in vitro* plasma protein binding of reference compounds has been performed. The data obtained from WP5 are now the basis for further biokinetic modelling.

In order to evaluate if toxicity is dependent on metabolism, the effects of 21 reference compounds are being been compared between a metabolic competent model (primary hepatocytes) and a non-metabolising cell type (HepG2) by use of MTT (WP6). 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. Recombinant-defective adenoviral vectors encoding major CYP genes involved in foreign compound metabolism have been developed as a way to overcome the problem with non-metabolising cell systems.

For Tamoxifen, Cyclosporin A, and to some extent also Tetracyclin, which toxicity is dependent on 3A4-dependent biotransformation, toxicity was observed only in HepG2 cells that were infected with adenovirus encoding for CYP450 3A4. Another aim of WP6 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 Work Package, WP 7.1, 26 chemicals (half of them neurotoxic) have been studied in native or differentiated human neuroblastoma SH-SY5Y cells, primary cultures of mouse or rat neurons, and mature re-aggregated rat brain cells by using more than 20 different endpoints (such as mitochondrial membrane potential, GABAA receptor function, [3H]GABA uptake, GAD activity, [3H]aspartate uptake, glutamate release, AChE activity, ChAT activity NMDA-glutamate receptor, acetylcholine esterase activity, uptake of [3H]noradrenalin, depolarisation- and carbachol-evoked changes in CMP, intracellular free Ca2+ concentrations, gene expression, ROS, 2-deoxyglucose uptake, [3H]uridine incorporation, [35S]methionine incorporation, GS activity, and CNP activity).

The results show that the broad collection of assays could predict the neurotoxic compounds. However, the challenge is to find more general assays that could identify several different neu-

rotoxic mechanisms of action. For the measurement of nephrotoxicity in WP7.2, transepithelial resistance (TEER) was chosen as the functional assay and the LLC-PK1 cell line as the test system. The REMS automated device was selected for measurement of TEER. Twenty-one reference chemicals (of which 5 are nephrotoxic) were tested and the overall results show that the TEER model using renal epithelial cells is a promising model for detection of nephrotoxicity.

The main goal of WP7.3 is 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 21 selected compounds using the MTT assay. Also, the following biochemical functions were examined in the cells: cellular ATP levels, formation of reactive oxygen species (ROS) as an index of oxidative stress, cellular protein content and mitochondrial membrane potential.

The results were compared with the MTT assays and suggest that neither ATP levels, ROS formation or protein content, when measured at early times (5 hours of incubation) or late times (24 hours of incubation) allowed a better discriminating effect than the one obtained by the MTT test. Another objective of WP7.3 is to develop an *in vitro* strategy to assess the effects of chemicals on the impairment of hepatocyte bile acids and bilirubin transport. This is done by generating a cell bank expressing the various hepatocellular transport systems and establishing assays as well as to characterise the interaction of chemicals *in vitro* with individual transporters.

As a result of these efforts, a protocol outlining a test system to identify (alert) for substances which are potentially hepatotoxic because of their capability to impair hepatic transport is expected.

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ARTEMIS

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

Contract No	LSHB-CT-2007-037862
Project type	SME-Specific Targeted Research Project
EC contribution	€ 1 984 900
Starting date	1 March 2007
Duration	36 months
Website	www.artemisproject.eu

Background and objectives:

The ARTEMIS project seeks to design, develop and optimise an *in vitro* system to replace the use of animals in transgenics and toxicology experiments, and in studies related to the effects of genes, chemicals, and neural tissue structure and function, such as memory and learning. From a scientific perspective, the ARTEMIS partners target the development of a three-dimensional neural tissue-like construct that is formed by the synaptic connections developed among neurons that are produced from mouse embryonic stem cells.

For its operational goal, the consortium seeks to replace transgenic animals with memory and learning deficiencies with the *in vitro* developed neural tissues. ARTEMIS will develop the tissue *in vitro* from embryonic cell lines that have the genes involved in memory and learning "switched off". The consortium will assess and compare the ability of the transgenic tissue to memorise electrical stimuli with that of the normal tissue.

Assessing the role of genes in memory and learning *in vitro* would provide preliminary information at tissue level, so as to determine the design of the transgenic animals towards the optimal ones with a higher probability of having altered phenotypes. This would effectively reduce the number of transgenic animals that are currently produced by trial-and-error methods. The proposed *in vitro* system can effectively integrate biochemical damages to behavioural ones.

Approach and methodology:

ARTEMIS is targeting the most critical network function of neural tissue: memory storage and learning capability. The functions concern the ability of the *in vitro* neuronal network to store in its pattern of connectivity the memory of one or two spatiotemporal electrical signals, and identify the difference between these signals and other stimuli in later stimulation (following memory storage).

The system in question would provide a broad technological basis, upon which a number of application-specific *in vitro* systems could be created. A neural tissue offering possibilities for the implementation of several types of *in vitro* tests is being developed. It is also significant that the *in vivo* system can provide a long-term and more direct assessment of toxic or pharmacological effects on neuronal development, i.e. learning impairment rather than end-point biochemical markers of RNA.

ARTEMIS will use the system in tests aimed at providing information on the biochemical mechanisms of memory defects in neural tissue produced from transgenic cell lines, for which flaws in earlier *in vivo* experiments had failed to refine the mechanisms. This system will also be used in neurotoxicity tests so as to measure the same system toxicity endpoints at different levels of the neural tissue organisation, such as cellular and synaptic network levels.

ARTEMIS

The design of the system refers not only to the network structure, but also to the process of the network development. It targets stimulus-specific connectivity patterns and includes structural and functional designs of the *in vitro* process of the neuronal network development. The patterns' features are not static; they evolve as the network is exposed to stimuli.

Expected outcome:

The proposed methodology of ARTEMIS, in connection with the scientific and technical advancements related to the project, will be effective in making feasible the training of a neuronal network that can respond specifically to electrical stimuli. Moreover, existing protocols of the neuronal differentiation of mouse embryonic stem cells provide evidence of suitability in the provision of neurons expressing genes involved in the formation of a functional synaptic network.

The consortium will draw up a proposal for formal prevalidation studies in order to ensure the validation and use of this system, and would guarantee that animals not be used in the prediction of behavioural changes.

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carcinoGENOMICS

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

Contract No	**LSHB-CT-2006-037712**
Project type	**Integrated Project**
EC contribution	**€ 10 440 000**
Starting date	**1 November 2006**
Duration	**60 months**
Website	**www.carcinogenomics.eu**

Background and objectives

Over a 5-year period, the carcinoGENOMICS project will 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. These tests will refer to a number of major target organs for carcinogenic action, e.g. liver, lungs and kidneys.

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). They will explore stem cell technology and generate genomic responses from a well-defined set of model compounds for genotoxicity and carcinogenicity. The project partners will assess phenotypic markers 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 genetic 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. Subsequently, predictive genetic pathways will be used as the scientific basis for developing high-throughput technology for accelerating analysis of genomics responses *in vitro*, indicating human carcinogenic risk, by a factor of 100. 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 Chemicals) 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.

It reflects the requirement of the LifeSciHealth Priority that alternative methods are to be developed and validated based on international standards. In doing so, these methods can obtain regulatory approval and then be applied in the industrial sector, as well as regulatory establishments and elsewhere.

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

carcinoGENOMICS

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.

Approach and methodology:

The major research hypothesis underlying this project is that it is possible to generate transcriptomic and metabonomic profiles from a set of well-defined genotoxic and non-genotoxic carcinogenic compounds in *in vitro* cellular systems, which reliably predict genotoxic and carcinogenic events *in vivo*. In order to test this hypothesis, the project has comprised different components in such a way so as to fully exploit the latest innovations in cell technology, genomics analysis, and bioinformatics:

- **Component 1:** innovative cell technology. Exploratory research will be conducted on human embryonic stem cells (hES) to determine whether it is possible to generate a robust and unlimited source of competent human cells. This represents an innovative approach and its feasibility will be investigated in the case of hES-derived hepatocytes, with a special emphasis on their propagation and differentiation.
- **Component 2:** novel combination of transcriptomic and metabonomic analyses of carcinogen-exposed cellular systems. Both transcriptomics and metabonomics are established genomic technologies, which have been shown to yield profiles capable of discriminating between classes of chemical agents.
- **Component 3:** novel applications for bioinformatics, with respect to standardising the infrastructure for high-quality data storage and mining.
- **Component 4:** dedicated novel, high-throughput technologies for speeding up the analysis of genomics responses to human carcinogens *in vitro*, based on discriminative genetic pathways for human genotoxicity and chemical carcinogenesis.

Expected outcome:

The outcome of the work foreseen under carcinoGENOMICS will be a mature protocol on toxicogenomics-based *in vitro* screens predictive for genotoxic and carcinogenic properties of chemicals which has been through pre-validation and which will be submitted to the European Centre for the Validation of Alternative Methods (ECVAM) for formal validation according to international standards. This will lead to subsequent regulatory acceptance and world-wide application in industry, regulatory establishments and elsewhere. This will be facilitated by including ECVAM as a partner in the management scheme of carcinoGENOMICS.

Combining pathway-associated gene expression with metabolic profiles generated *in vitro*, as is foreseen in carcinoGENOMICS, represents a highly innovative approach possibly leading to *in silico* models that may be used to predict the carcinogenic potential of a compound *in vivo*. Furthermore, toxicogenomics-based assays may outperform currently available tests for genotoxicity, as well as for genotoxic and non-genotoxic carcinogenicity, without using animals. These *in vitro* methods may therefore play a major role under the new system of the Community regarding the REACH initiative.

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COMICS

Comet assay and cell array for fast and efficient genotoxicity testing

Contract No	LSHB-CT-2006-037575
Project type	SME-Specific Targeted Research Project
EC contribution	€ 3 189 385
Starting date	1 January 2007
Duration	36 months
Website	http://comics.vitamib.com

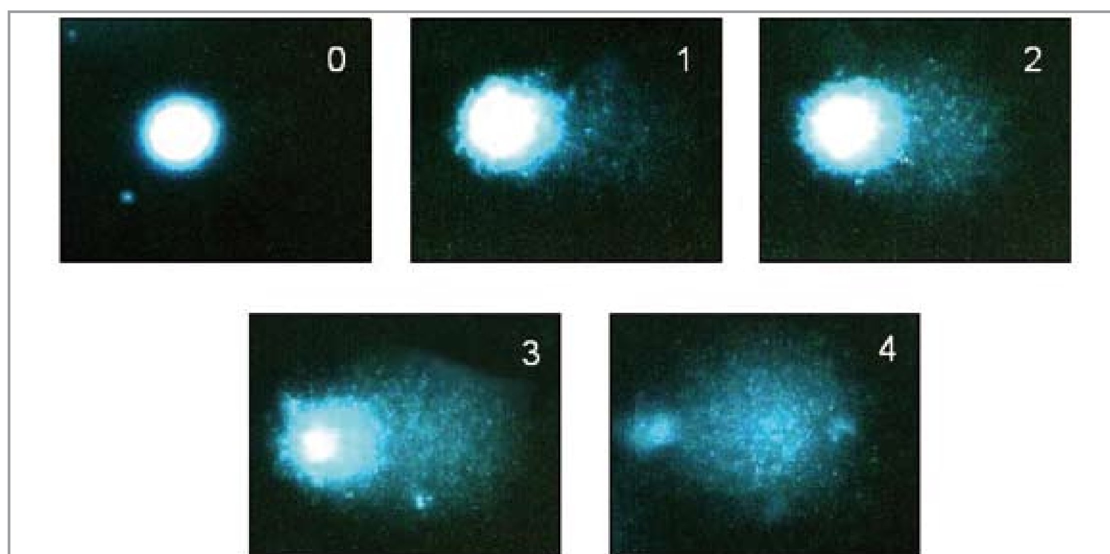
Background and objectives:

A battery of reliable and validated *in vitro* assays is needed to test for genotoxic and cytotoxic effects of chemicals, without resorting to animal experiments. The comet assay, a sensitive indicator of DNA damage, will be combined in this project with the Cell Array system in order to establish and validate high capacity assays suitable for chemical testing. Up to 800 cell samples will be processed for comets on a single microscope slide. Arrays will use cells with different metabolic capabilities, and data on cytotoxicity will be obtained in parallel with DNA damage. A medium-throughput assay will also be developed.

Approach and methodology:

Comet analysis by differential staining of damaged/undamaged DNA using established and novel dyes combined with automated image analysis will be faster and more reliable than at present. A crucial aspect of the cellular response

Figure 1. The comet assay: examples of comets from cells with increasing levels of DNA damage (0-4). The images are produced by embedding cells in agarose on a glass slide, lysing to remove membranes and soluble proteins, including histones, and conducting electrophoresis at high pH. DNA breaks relax supercoiled DNA loops which are then able to extend towards the anode and form the 'comet tail'. The % of DNA in the tail quantitatively reflects the number of DNA breaks. [Reproduced, by permission of Humana Press, from Molecular Biotechnology 26 (2004) 249-261]



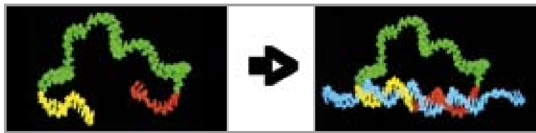


Figure 2. The principle of the 'padlock probe'. The probe (left panel) has at its ends sequences complementary to adjacent parts of the target DNA. After hybridisation, these ends are ligated and the probe thus locked onto the target. These probes can be designed to lock onto defined gene sequences, and so permit an examination of damage and repair in those specific genes. [Figure provided by Prof. Mats Nilsson.]

to DNA damage is DNA repair; variations between people can affect cancer risk, while genotoxic chemicals can act by interfering with repair. COMICS will compare two methods for measuring repair, one based on the comet assay and the other using a 'Repair Chip' approach. In addition, the project partners will apply fluorescent probes which lock onto the DNA after hybridisation ('padlock probes') to study gene-specific DNA repair.

COMICS will also assess the modified comet assay methods for reproducibility and sensitivity in an inter-laboratory prevalidation trial, using a coded set of standard chemicals, to satisfy regulatory bodies, as well as industrial users of the technology. The result will be robust, validated, high-throughput *in vitro* genotoxicity tests. This proposal brings together academic partners and SMEs, together with a large industrial concern, and the European Centre for the Validation of Alternative Methods (ECVAM). Dissemination through professional and trade publications, regulatory channels, and scientific conferences will lead to widespread adoption of the new methods.

The comet assay for DNA damage is widely used but has a major drawback as it is labour-intensive, and therefore can be used only in studies in which the numbers of samples are relatively small. The development of high-throughput variants will increase its applicability in both genotoxicity testing and population biomonitoring. DNA damage is a good marker of exposure to genotoxic chemicals; measuring cellular repair of this damage gives additional information on the mode of action of genotoxic agents and on individual susceptibility to carcinogens.

The overall objective is to develop reliable and tested genotoxicity and cytotoxicity assays that will, when combined, reduce the need for animal experiments in assessing the safety of chemicals. Specific aims include the following:

- increasing the throughput of the comet assay by up to 20 times;
- developing further the cell array system as a parallel assay for cytotoxicity;
- seeking optimal cell types for use in genotoxicity and cytotoxicity testing;
- increasing the speed of scoring of comets;
- using lesion-specific enzymes and inhibitors to measure different kinds of DNA damage;
- developing and comparing methods for measuring DNA repair activity;
- devising an approach to measure gene-specific DNA damage and repair;
- validating the comet assay in its various forms;
- establishing reference and internal standards for use in the comet assay;
- making the various innovative products available for use by companies and researchers investigating DNA damage and repair.

Role of SMEs:

The SMEs target the following actions:

- devising new software for image analysis as required by the novel methods of scoring of comets;
- providing suitable cell lines;
- offering custom-made DNA-damaging chemicals for calibration purposes;
- providing dyes for differential staining of

- intact and damaged DNA;
- pre-market product development and testing.

Expected outcome:

COMICS seeks to develop validated assays for DNA damage and repair, that will be accepted by regulatory authorities for use in genotoxicity testing and will thereby minimise reliance on experimental animals.

Potential applications:

Commercial genotoxicity testing, biomonitoring in human population studies, basic research in DNA damage and repair.

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EXERA

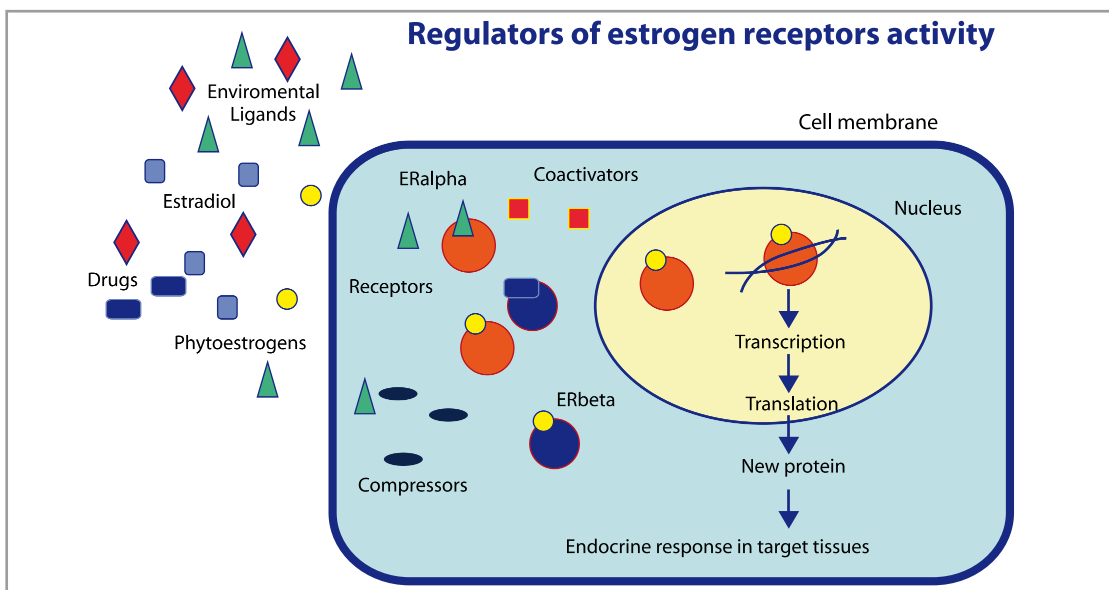
Development of 3D *in vitro* models of estrogen-reporter mouse tissues for the pharmaco-toxicological analysis of nuclear receptors-interacting compounds (NR-ICs)

Contract No	LHSB-CT-2006-037168
Project type	Specific Targeted Research Project
EC contribution	€ 2 173 492
Starting date	1 October 2006
Duration	36 months
Website	www.altaweb.eu/exera

Background and objectives:

Industries from different fields (pharmaceuticals, chemicals, cosmetics, foods and toxicologicals) need reliable, fast and economic *in vitro* models, which are alternative to animal testing and can provide predictive data on the actions of NR-ICs, and in particular ER-ICs (estrogen receptors-interacting compounds), on animal and human health. These needs are increasing for a number of reasons:

- more new drugs with endocrine action are being produced (mainly estrogens/antiestrogens for women), and 80% of the marketed drugs is directed to women: birth control, cancer therapy and prevention, hormone replacement therapies;
- the recognition that a wide and increasing number of xenocompounds that come from the industrial production and that contaminate the environment are active endocrine structures (for both men and women);
- research evidence is increasingly emphasising the limitations of several non-mammalian models for a correct estimation of the physiological consequences (risks and benefits) linked to the exposure of humans to ER-ICs.



EXERA

The need for appropriate *in vitro* models that can reproduce features and reactivity of specific mammalian target tissue/organs to ER-ICs is thus becoming an imperative research priority. The scientifically, economically, socially and ethically relevant stakes are considerable.

The tissue- and organ-specific *in vitro* models that have been built so far have several serious limitations:

1. Most of the available cell lines of mammalian origin are derived from tumours or have a transformed phenotype. Their functional and structural features do not mirror the original tissue, thus giving rise to an altered response to various endogenous and exogenous factors with respect to the *in vivo* situation (Joung K, 2003; Lewis JB, 2000; Van Den Belt K, 2004).
2. When mammalian-derived *in vitro* models are available, they consist of primary cell cultures or in isolated tissue slices: their *in vitro* survival is limited (time-course and dose-response studies are very difficult) and their behaviour is strictly dependent on the variability among individuals (their performance cannot be standardised). Moreover, if of human origin, they have the disadvantage of depending on regular supplies from available clinical sources.
3. 2-Dimensional culture conditions may be not optimal for tissue-like organisation and cellular functions (e.g. polarised cells of a parenchymal tissue, which normally require complex cellular interactions, cannot behave physiologically when adhering to solid substrates, as in the case of conventional culture conditions).
4. Conventional cell cultures often do not express suitable easy-to-assay quantifiable markers or they need transfection procedures that increase the variability of the data. The most commonly used cell cultures to study endocrine actions are strongly biased toward female-specific effects, being mostly composed of female cells (i.e. endocrine responsive cancers) (Kojima M, 2005). The recent knowledge of the presence of high concentrations of estrogen receptors in male tissues makes these biological materials obsolete for some aspects.
5. The systems used for the *in vitro* and *in vivo* analysis of NR-ICs (mainly estrogens and androgens) are generally composed of cells derived from reproductive tissues. The recent knowledge of the widespread distribution of nuclear receptors (in particular steroid receptors) in all the tissues of the organism and their involvement in several diseases, makes the available systems inadequate to assess the effects of NR-ICs on the whole physiology (Villa R, 2004).
6. The available models do not easily provide information on the effects of compounds at different developmental stages.

To overcome the above-mentioned deficiencies of inadequate *in vitro* models, seven industry representatives have decided to join their efforts with those of three public research organisations in order to develop novel *in vitro* 3D models of estrogen-reporter mouse tissues for the Pharmaco-toxicological analysis of ER-ICs for liver, skin, bone (non reproductive systems), and ovaries and testis (sex-specific reproductive systems).

Approach and methodology:

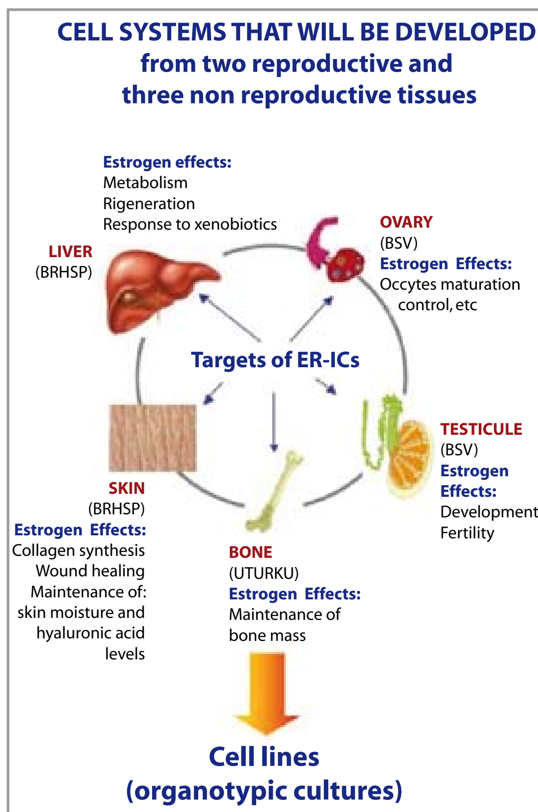
EXERA's approach is based upon powerful evidence:

Use of the transgenic MOUSE-1: This estrogen-reporter mouse model represents a new strategy that allows studying estrogen receptors-mediated gene regulation *in vivo* and in derived *in vitro* systems. The numerous studies performed on this animal model in several laboratories have demonstrated its reliability and suitability to the

study of molecule acting through estrogen receptors. The mouse was generated by Partner 2 (Maggi A, 2005; Ciana P, 2004; Di Lorenzo D, 2002) and is the object of a patent application (PCT N° WO 02/01949 A3 date 10.01.2002) extended to several extra-European States.

3D cultures: 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.

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



Cell isolation from tissues of MOUSE-1: Reliable protocols available inside the partnership will be applied so as to isolate well differentiated cells from liver, skin, bone, testis and ovaries of MOUSE-1 and establish cell cultures with "physiological" estrogen-dependent phenotypes for immortalisation. Cell cultures will be constantly controlled and characterised with respect to the *in vivo* situation with specific markers (luciferase enzymatic assay and *in vitro* cell imaging, endogenous markers of tissue health and differentiation) and by gene expression profiles. Cells will be isolated either from male and female mice.

Conditional Immortalisation: This step will be performed by transfection methodologies in 3D and by using suitable commercially available vectors, made inducible by specifically modified antibiotics devoid of hormonal actions. The immortalising gene will be switched on for cell production, and off for characterisation and testing.

Constitution of a cell bank: Immortalised cell cultures that will satisfy the following parameters will be expanded and controlled for banking: (1) Immortalisation must not modify the estrogen-dependent pathways. This will also be controlled by gene expression profiles; (2) Tissue health and homeostasis. Cells must show a healthy differentiated phenotype with respect to tissue specific markers.

3D-cultures adapted to grow cells with an unaltered estrogen-dependent phenotype: Estrogen-dependent pathways will be characterised in 3D-cultures. Comparison between hormone stimulation *in vitro* and *in vivo* will be performed with the use of the transgenic marker (luciferase) and gene expression profiles. Data on the activity of selected ER-ICs will be produced in each specific cell line with respect to: (1) agonism/antago-

nism of estrogenic actions; (2) tissue specificity and gender specificity.

Assessment of the 3D-culture systems for the pharmaco-toxicological characterisation of ER-ICs.

Expected outcome:

- the application/adaptation of new 3D-culture technologies to cell cultures devoted to the study of ER-ICs;
- the constitution of a cell bank;
- a battery of differentiated 3D cell-based systems derived from estrogen-reporter mice for basic and applied research (e.g. pharmacology and toxicology), public use and industrial use.

Major publications:

Penza, M., Montani, C., Romani, A., Vignolini, P., Ciana, P., Maggi, A., Pampaloni, B., Caimi, L., Di Lorenzo, D., 'Genistein accumulates in body depots and is mobilized during fasting, reaching estrogenic levels in serum that counter the hormonal actions of estradiol and organochlorines', *Toxicol Sci*, 2007, Mar 3.

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

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Invitroheart

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

Contract No	**LSHB-CT-2007-037636**
Project type	**Specific Targeted Research Project**
EC contribution	**€ 2 701 611**
Starting date	**1 January 2007**
Duration	**36 months**
Website	**http://er-projects.gf.liu.se/~invitroheart**

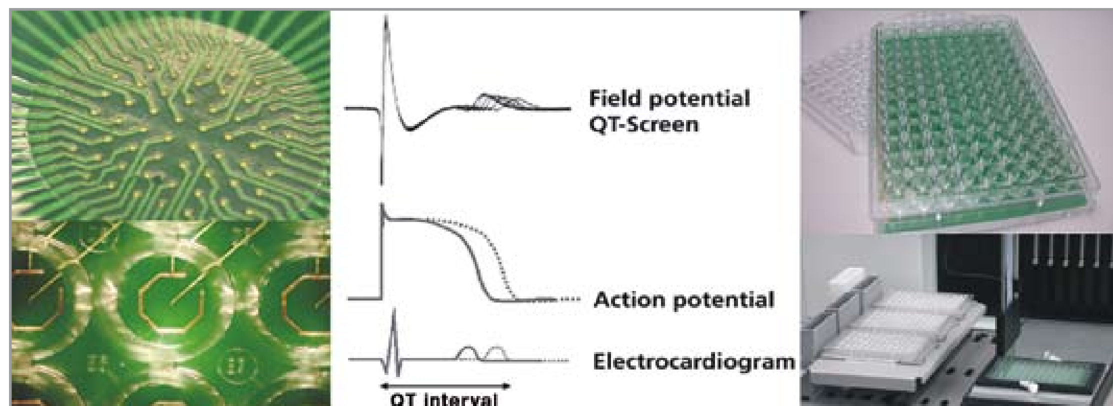
Background and objectives:

Invitroheart seeks to establish stable cell lines that reflect human cardiomyocyte properties in a reliable manner, through the development of models derived from human embryonic stem (hES) cells. Its aim is to deliver reliable *in vitro* models that the pharmaceutical industry could use to replace experimental animals, in the following instances: (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 in replacing current techniques with animal experimentation in the selection and optimisation of lead compounds, and in the documentation of a selected drug candidate before it enters clinical phases. In the toxicity testing of chemical substances, the replacement of animal testing methods can be attained as well. The means to accomplish the objective, in addition to new stable hES cell-de-

The QT-screen technology for assessment of electrophysiological effects

In the left panel, the transition from the 60 electrode Multi-Electrode-Array 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 from this technology with illustrations of corresponding action potential and electrocardiogram measurements, under control conditions and with prolonged QT interval. The right panel shows an automated system for high throughput QT prolongation screening. This QT-screen technology can thus replace telemetry/electrocardiogram studies on dogs and other animals, in order to assess effects on cardiac repolarisation and arrhythmogenic activity of drugs (QT-screen technology from Multi Channel Systems GmbH).



Invitroheart

▲ Cell culture plates with optical sensor system for oxygen and pH monitoring

Monitoring of the important physiological parameters pH and oxygen is essential for assessing the physiological condition of a cell culture. Consequently, this also becomes a very 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 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 equipped with OxoDishTM and HydroDishTM technology for oxygen and pH monitoring respectively, and on the right a SensorDishReaderTM monitoring device (technologies from PreSens GmbH).

rived cardiomyocytes, are as follows:

- state-of-the-art methods for electrophysiological cardiac cell monitoring;
- optical micro-sensor monitoring in micro-cultivation systems for *in vitro* screening;
- a multi-micro-bioreactor platform for high-throughput screening of drugs and chemicals.

Approach and methodology:

Invitroheart will carry out comparative studies of cardiomyocytes derived from hES cells with established *in vitro* models in order to validate the new models and methods. The outcome of the project is new efficient *in vitro* prevalidation models that will significantly reduce the use of animal experimentation for cardiotoxicity testing by 60% to 80%. 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.

Studies of toxicity and safety pharmacology in general and cardiotoxicity in particular are key activities throughout drug discovery programmes in the pharmaceutical industry. Such activities are initiated to detect detrimental compound effects. For example, electrophysiological changes such as QT prolongation, which leads to delayed ventricular repolarisation and cardiac arrhythmia, are induced by a vast range of chemical entities for multiple therapeutic areas. Unintended QT effects of new drugs are the most common cause of drug withdrawal from the market and delays in or lack of regulatory approval for marketing.

Clinical cardiotoxic effects are defined as symptoms of clinical heart failure, and subclinical cardiotoxic effects as cardiac abnormalities detected in asymptomatic persons by means of various methods. One of the problems is the availability of preclinical models capable of rapidly screening a large library of substances and of providing rational bases for clinical trials. A significant bottleneck in the development of novel assays has been the lack of research purposes. Animal models have been invaluable for risk assessment of compound safety; however, critical limitations remain in these models for robust prediction of certain toxic outcomes in humans.

Aim:

The overall aim of the project can be summarised as follows:

- to replace animals with human cell culture systems, in preclinical pharmaceutical development and chemical substance toxicity testing;
- to support the predictability of the drug discovery and development process of cardiovascular pharmaceuticals by allowing more reliable and relevant testing in the preclinical phase, and hinder weak lead candidates from entering clinical phases with innovative human cardiomyocyte cell systems;
- to deliver an *in vitro* testing system with adjacent methodology pertinent for validation in GLP/SOPs environment for cardiac safety;
- to deliver *in vitro* testing systems with adjacent methodology pertinent for chemical substance toxicity testing within REACH;
- to reduce or even totally abolish (this being the ultimate aim) the use of animals in drug and chemical substance testing, refine the model system under consideration and replace the animal models currently used.

Specific technology-related objectives are as follows:

- to establish relevant hES cell-derived cardiomyocytes cultures that allow a more predictable preclinical lead testing programme to be carried out;
- to develop a real-time sensor-based *in vitro* model for short-term studies of cardiac side-effects, that mimics the function and complexity of the cardiomyocyte tissue *in vivo*;
- to develop a real-time sensor-based *in vitro* model for long-term studies of cardiotoxicological effects, that mimics the function and complexity of the cardiomyocyte tissue *in vivo*;
- to establish a versatile cell lab platform based on the developed cell lines, and cultivated in advanced miniaturised bioreactor systems with non-invasive measurement techniques for *in vitro* testing of cardiotoxicity.

A monitoring platform for high-throughput screening

An important aim of Invitroheart is to integrate several bioassay technologies, such as QT-screen and optical sensors, into one monitoring platform with hES cell-derived cardiomyocytes. This platform technology will enable assessment of cardiac toxicity at a far greater speed as compared with conventional animal models currently used in the pharmaceutical industry.

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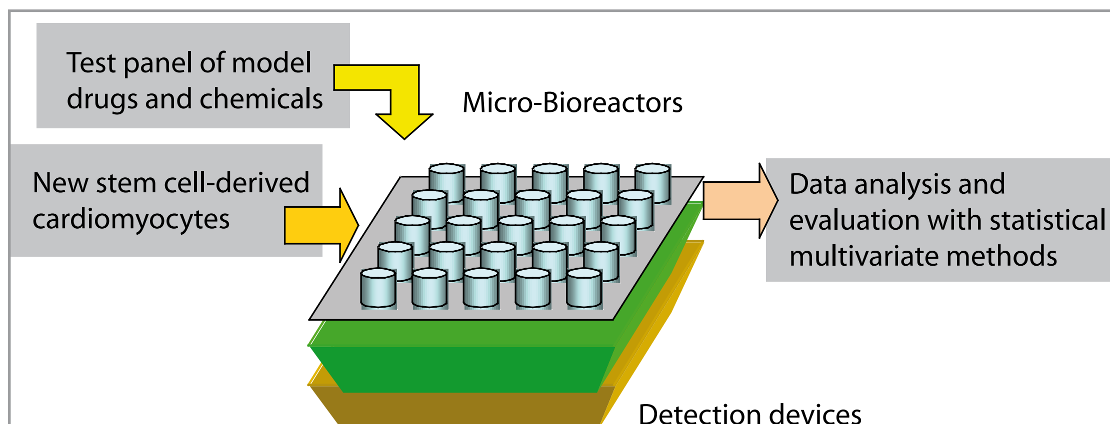

Invitroheart

Role of SMEs:

Out of nine partners in Invitroheart, four are SMEs (Cellartis, Multi Channel Systems, Pharmacelsus and PreSens) and their share of the requested funding from the Commission amounts to 59%. The SMEs' contribution is crucial to the project as regards the generation of cells and assay technologies, and as experts in life science and microsensor technologies, they are the key providers of state-of-the-art technology.

Expected outcome:

Successful results, if adopted by the European pharmaceutical community, will lead to:

- the preservation of significant numbers of experimental animals;
- reduced work carried out during pharmaceutical drug discovery and development in the preclinical and early clinic phases;
- improved predictability of quality of lead candidates, increasing the chances for passing the entire clinical trials process;
- reduced total development time of leads;
- attainment of better quality and safety in documentation filed to regulatory bodies.

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LIINTOP

Optimisation of liver and intestine *in vitro* models for pharmacokinetics and pharmacodynamics studies

Contract No	**LSHB-CT-2006-037499**
Project type	**SME-Specific Targeted Research Project**
EC contribution	**€ 2 933 291**
Starting date	**1 January 2007**
Duration	**36 months**
Website	**www.liintop.cnr.it**

Background and objectives:

The main aim of LIINTOP is to optimise and provide established protocols and experimental *in vitro* models for testing intestinal and liver absorption, metabolism and toxicity of molecules of pharmacological interest. The added value of the project, with respect to the existing experimental approaches in this field, is to provide optimised sequential procedures that are easily amenable to validation studies for the screening and testing of new drugs, possibly by miniaturised and automated technology. The direct participation of SMEs in the research activities will assure that those procedures will meet the requirements of industrial application.

The project, therefore, comprises a number of different approaches, which will integrate at various levels. A first basic approach will be the optimisation of *in vitro* liver and intestinal models for their use in the transport and toxicity of structurally diverse reference drugs, chosen with the help of a steering committee of relevant stakeholders. A parallel approach will deal with the identification of the transport and metabolic pathways, and possible cytotoxic effects of these drugs, in order to develop appropriate monitoring procedures. Newly advanced technologies (genomics, proteomics, metabolomics) will be used to develop high-throughput models related to the specific area of intestine-liver absorption and biotransformation. Each approach will address the reliability of the protocols involved and the relevance of the whole procedure to the *in vitro*/*in vivo* extrapolation of drug effects. To this end, a unit in charge of computer-based studies will support and pilot the project throughout its course.

In Europe, three directives regulate the testing of chemicals: Council Directive 67/548/EEC and its subsequent amendments; Council Directive 88/379/EEC and subsequent amendments, and Council Directive 76/769/EEC. Moreover, Council Regulation No. 793/93 on the evaluation and control of risk of existing substances also deals with the same topic. The main new aspect is that a distinction has been made between the new substances identified since 1981, and those identified before then. For the latter group (containing about 100 000 substances), it has been estimated that insufficient data are available concerning their safety. Thus, the White Paper proposes a harmonisation of testing requirements for new and existing substances, by introducing a new system for the Registration, Evaluation and Authorisation of new and existing Chemical Substances (REACH) (COM-2001/88 Final; COM-2003/644).

This implies, on the one hand, a cumbersome plan of testing, and on the other hand, the use of an extensive number of animals. The European Centre for validation of Alternative Methods (ECVAM) has already addressed the possibility of using alternative methods, based on the 3Rs model, in order to reduce this number, or at least to reduce the animal suffering associated with certain kind of tests. The EU has addressed this is-

sue in Directive 86/609. The use of non-validated alternatives has also been suggested, based on the 'weight of evidence', i.e. widely used and well-consolidated procedures. This project may well contribute to those aspects, by providing new procedures submitted to optimisation.

A successful outcome of LIINTOP, in fact, will have a strong and diversified impact on social and economic issues. The main effect will be related to the expanded use of *in vitro* systems, and to meeting existing expectations from the public at large and from industrial enterprises. *In vitro* systems meet with the demand for reducing animal experiments, thus satisfying a widely shared ethical concern. Furthermore, they offer economic advantages in terms of reduced time consumption and lower costs in the assessment of safety for novel drugs.

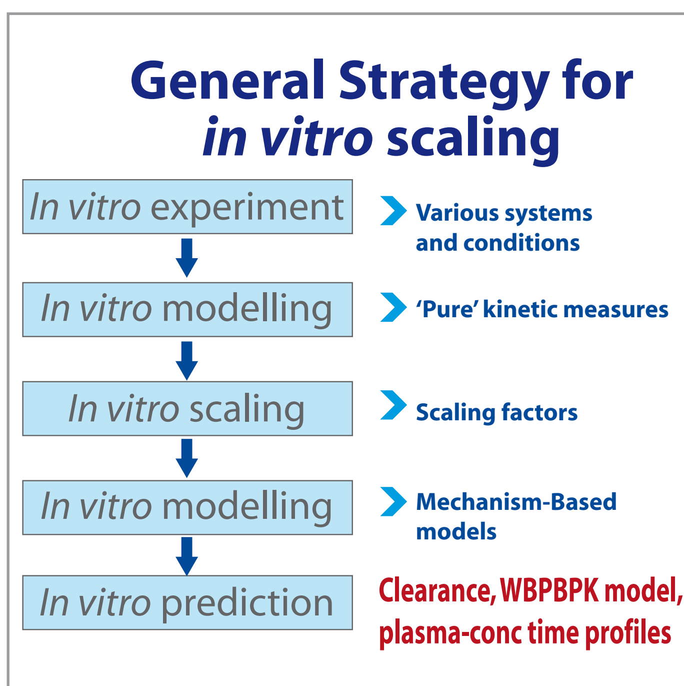
From the scientific point of view, the LIINTOP project represents a unique effort to link, by an *in vitro* approach, different systems (i.e. gastrointestinal tract and liver) and pathways involved *in vivo* in the absorption and metabolism of orally ingested substances. This kind of approach is important for further development of tests for chronic exposure in which the interrelation between different organs is a key factor, and where at present nearly no *in vitro* data are available. Such integrated models will be developed to the point of entering pre-validation procedures in order to be submitted to regulatory boards.

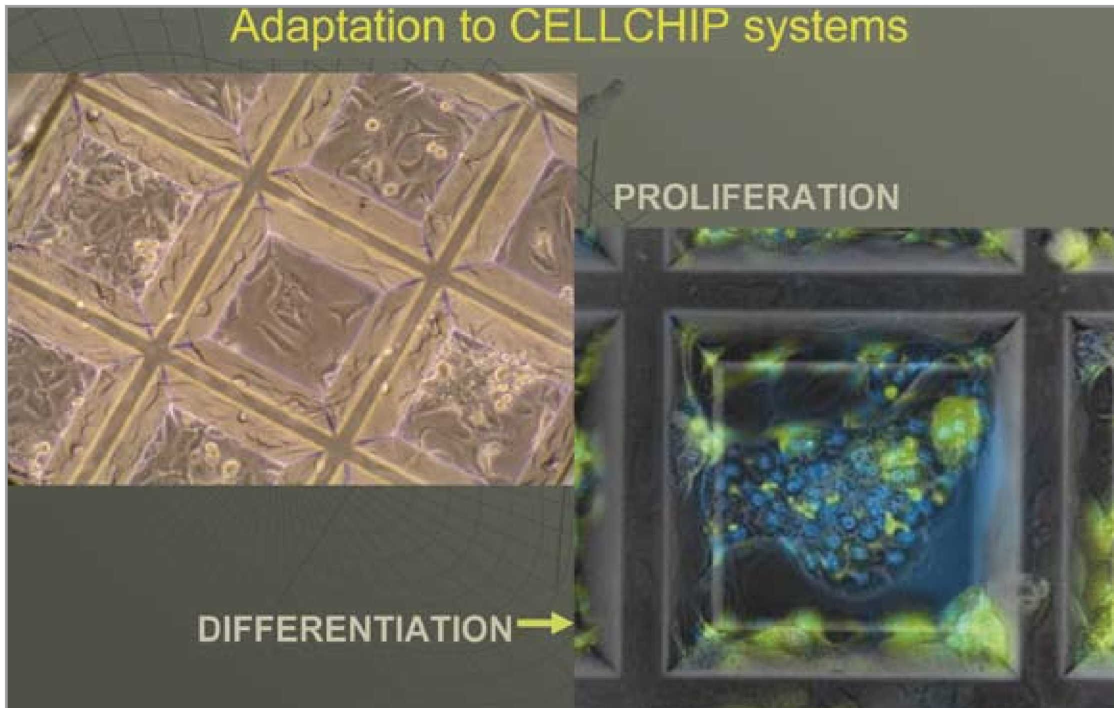
Role of SMEs:

The direct participation of SMEs in the research activities (particularly on transporters and metabolites identification) will ensure that the optimised sequential procedures for the screening and testing of new drugs will meet the requirements of industrial application. This proposal addresses an issue of great relevance in the drug discovery process, i.e. absorption and enterohepatic bio-disposition of drugs, which presently still relies on *in vivo* experimentation. The development of integrated *in vitro* predictive tools capable of addressing these issues — possibly sustained by miniaturised and automated technology — will have a positive outcome in the competitiveness and success of the pharmaceutical industry, since decisions taken on partially characterised compounds (or those with questionable human relevance) may lead to great economic losses at later stages.

Expected outcome:

LIINTOP seeks to provide standardised cellular models of human hepatocytes and enterocytes reliable for the prediction of drug absorption,





Human HepaRG hepatocytes differentiated in cellchip system (from C.Guguen-Guillouzo, Inserm U522, Rennes, France)

metabolism and toxicity. Sequential procedures, easily amenable to validation studies, possibly by miniaturised and automated technology, will be developed. Based on these models, the project partners will obtain a database concerning *in vitro* absorption, metabolism and toxicity of selected drugs, including the characterisation of the regulation of relevant genes. They will develop prediction mathematical models of pharmacokinetics and pharmacodynamics, based on the available *in vivo* data and on the *in vitro* data from the cellular models used in the project.

Best case scenario:

- transferring the best hepatocyte and enterocyte models and relevant testing procedures to the industrial setting, for high-throughput applications in the development of new drugs;
- establishing the relevance of the proposed *in vitro* models to the human *in vivo* situation.

Potential applications:

Optimised sequential procedures and reference standards concerning *in vitro* models of hepatocytes and enterocytes, easily amenable to validation studies, will allow a new molecule of pharmacological activity to be tested for the following:

- potential toxicity at the level of the intestinal mucosal barrier;
- intestinal absorption;
- metabolic modification in the intestinal cells;
- absorption and metabolism in the hepatocytes;
- hepatotoxicity;
- optimal pharmacokinetic behaviour.

LIINTOP

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MEMTRANS

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

memtrans

Contract No	LSHB-CT-2006-518246
Project type	Specific Targeted Research Project
EC contribution	€ 1 900 000
Starting date	1 April 2006
Duration	36 months

Background and objectives:

MEMTRANS seeks to optimise and prevalidate *in vitro* cultured cell models so as to predict oral absorption and pharmacokinetics of efflux systems substrates (e.g. P-gp, MRP2, BCRX), as well as to establish an *in vitro* cut-off for the risk of secretion-associated problems and to study the structure-affinity-transport relationships. These actions would allow the following:

- a reduction in the number of animal experiments to study efflux processes;
- the *in vitro* study of drug-drug (including phytopharmaca) and drug-food interactions related with secretion transporters;
- the gathering of useful information for modelling transporter interactions in other normal and transformed tissues (blood brain barrier, lung mucosa, tumours).

The objectives of the project will be achieved by using a systems biology approach that involves modelling and simulating the complex dynamic interactions between proteins (transporters), metabolites (i.e. substrates) and cells (lipoidal barriers). Computational and mathematical predictive models will be generated from the data, based on the system parameters and on the drug characteristics. The experimental data generated in the project will be analysed from a mechanistic point of view in order to split all the individual steps involved in transport. The mathematical models, in combination with the right physiological, physicochemical and chemical information, could be applied to predict drug transport in a wide range of membranes in the organism (blood brain barrier, liver, kidney, tumours, etc.), but especially in the gastrointestinal tract.

Approach and methodology:

The MEMTRANS partners target the following items.

- Prevalidation of different methods to predict gastrointestinal absorption of secretion transporters substrates.
- Standard operation procedures for maintaining and working with the developed models in order to reduce inter-laboratory variability and to allow exchange of data obtained in different laboratories. This might lead to a reduction of animal and human studies, as shown by the validation of cell culture models for gastrointestinal absorption, which is accepted by the FDA in its guidance about biowaivers based on the Biopharmaceutical classification system. These concepts are also included in the EMEA bioequivalence guidelines.
- Mathematical models to predict from *in vitro* data the *in vivo* intestinal efflux characteristics and its impact in drug ADME.

Expected outcome:

With this improved validated *in vitro* methodology, avoiding extensive animal experimentation

MEMTRANS

in preclinical phases to study efflux processes and to prevent failures of drug candidates due to poor absorbability would be possible. At the same time, an appropriate *in vitro* model with a level expression of P-glycoprotein — similar to that found in experimentation animals and with less interlaboratory variability — would allow the combination of data obtained in different laboratories. In this way, it will improve the statistical signification of the established relationships. All these facts will be reflected in the faster development of new drugs for patients.

The oral route for drug administration is the most convenient and preferred by patients, and the prediction of drug absorption from the GI tract is a key issue for a potential new medication in the process of drug design and development. The early prediction of favourable pharmacokinetic properties is the new paradigm in the screening of drug candidates. Current *in vitro* models for the predictions of drug transport across biological membranes include cell cultures that reproduce physiological characteristics of different barriers, such as the intestine, the blood-brain barrier or the kidney and liver. These models have shown good performance for compounds that are transported by passive diffusion. However, for drugs with a carrier-mediated mechanism, the predictions are less accurate, mainly due to the differences in expression levels and affinity for the carriers *in vitro* versus the *in vivo* situation.
It is of paramount importance to know in the early stages of drug discovery, whether a new compound is or is not a substrate and/or an inhibitor of an efflux pump, in order to prevent absorption problems, drug-drug, and drug-food interactions, and to minimise pharmacological resistance.

The *in vitro* cell cultures currently used (as Caco-2 cells) for high-throughput screening of absorbability-distribution of new drugs show good performance with passively absorbed drugs, but in the case of compounds absorbed by active mechanisms the predictability is still poor. The expression level of the transporters differs between clones and laboratories, and it is highly dependent on the culture conditions. Standardised procedures and the use of quality controls to verify the functionality of the transporters are essential for achieving good predictive performance. On the other hand, stable cell lines with good expression of the transporter of interest are scarcely available.
The MEMTRANS team will collaborate with the BIOSIM Network of Excellence in the dissemination tasks. Therefore, the MEMTRANS results will be disseminated within the BIOSIM as a complementary channel.

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PREDICTOMICS

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

PREDICTOMICS

Contract No	**LSHB-CT-2004-504761**
Project type	**Specific Targeted Research Project**
EC contribution	**€ 2 259 754**
Starting date	**1 September 2004**
Duration	**39 months**
Website	**www.predictomics.com**

Background and objectives:

Over 30% of drugs under development have failed in the last decade because of mid-term toxicities in animals, or as a result of unexpected adverse events in early clinical studies that could not be detected in preclinical studies. The prediction of toxicity of this class of compounds would be more efficient if good quality and human-relevant toxicological data could be made available at earlier development stages.

While acute toxicity can be reasonably detected during the early preclinical studies, long-term toxicity is more difficult to predict, relying almost exclusively on animal experiments that frequently are not predictive enough for humans. Animal experimentation of this kind is expensive, time-consuming, raises ethical issues and does not necessarily imply toxicological relevance to man.

Despite past efforts to develop predictive *in vitro* tests for specific organ toxicity (i.e. isolated/perfused organs, tissue slices, primary cultures, cell lines, organotypic cultures, etc.), their reliability in anticipating chronic toxicity is still very limited.

There is a clear need for well-standardised and robust *in vitro* models suitable for the screening of toxic and sub-chronic toxicity induced by xenobiotics. The underlying concept in PREDICTOMICS was that chronic toxicity phenomena may be identified even in short-term assays if proper indicators are identified and monitored.

This Specific Targeted Research Project aimed to develop an *in vitro* strategy predictive of drug-elicited kidney and liver chronic toxicity, by combining emerging technologies and advanced culture models.

As a combined basic and targeted research project, PREDICTOMICS aimed for the following:

- a better understanding of the mechanisms of chronic toxicity to identify relevant and early changes induced by chronic toxins;
- the development of advanced and innovative cell culture models for liver and kidney cells, including targeted-directed cell transformation and stem cell technology;
- optimisation and technological improvement of genomic, proteomic and cytomic tools to increase their sensitivity and accuracy for these types of toxicity studies;
- identification of primary (mechanistically linked) and secondary biomarkers of chronic toxicity, based on combined genomic proteomic and cytomic analysis of cells exposed to model hepatotoxins and nephrotoxins;
- based on the identified primary and secondary biomarkers, the establishment of a hierarchical flow chart and a mathematical model so as to anticipate a potential risk of causing chronic liver and/or chronic toxicity of new drug candidates;
- prevalidation of the screening platform.

PREDICTOMICS

Approach and methodology:

The strength of this project lies in the combination of a comprehensive analysis of cell effects induced by toxicants via Cytomics/ Genomics/ Proteomics (i.e. PREDICTOMICS), and a deeper mechanistic knowledge of drug-induced chronic toxicity. Outstanding among these were the identification of endpoints/pathways affected by the early toxic compounds treatment, and the identification of primary and secondary biomarkers sets, classified according to their relevance to the mechanism of chronic toxicity.

Main findings:

The following publishable results were obtained in Years 1 and 2 of the project, and are sustained by papers, either published or submitted.

1. The suitability of different hepatocyte cultures for predictomics analysis was investigated. Collagen cultures were also examined, concerning their ability to maintain long-term cultures, and simultaneously to meet the requirements for further genomic analysis. Studies on the role of gene expression and regulation of hepatocyte adult phenotype were conducted using trichostatin analogues and vectors encoding for key transcription factors (P1, P3). Primary rat hepatocytes treated with HDAC inhibitors better retain the adult phenotype in culture, and may constitute a suitable strategy to improve performance of primary cultured hepatocytes.
2. The role of transcription factors (HNF4), activators, as well as repressors (SRC1, SRC2, PGC1 alpha, PCAF) were investigated in detail, to understand the lack of expression of CYP's in the human hepatoma HepG2. Adenoviral expression vectors were constructed allowing a detailed analysis of these factors. Expression of PGC1 alpha in hepatoma cells resulted in the upregulation of key hepatic genes, including CYPs.
3. Hepatic progenitor cells were successfully isolated from the human liver in order to derive hepatocytes suitable for the purpose of the project. In an appropriate differentiation culture medium, the differentiation of progenitor cells towards the two lineages (ductal, parenchymal) was achieved. Cells acquire many of the biochemical features displayed by adult hepatocytes, but showed limited proliferation/passage stability.
4. A methodology to examine multiparametric hepatocyte metabolism indicators was set up and adapted for rapid FACS analysis of hepatocytes treated with drugs.
5. It was possible to reproduce *in vitro* the esteatosis induced by drugs. The genomic analysis allowed the identification a number of genes and proteins that are consistently upregulated as a consequence of the lipid accumulation in hepatocytes. A cytomic analysis was also established, enabling an accurate measurement of lipid content in hepatocytes treated with steatosic drugs. The combination of the three '-omics' technologies appeared to be a suitable integrated strategy to identify this class of hepatotoxicants, in accordance with the PREDICTOMICS principles.
6. To approach the identification of drugs with cholestasic potential, new fluorescent bile acid derivatives were synthesised and successfully adapted to cytometric analysis. Initial studies with model cholestasic drugs revealed effects that could be monitored with this assay. Effects on gene expression in hepatocytes were also conducted; however, no conclusive results have yet been obtained, as the number of genes altered is small.
7. Optimised, stable, and well-characterised renal mono- and co-cultures for *in vitro* nephrotoxicity studies were established,

and standard operating procedures prepared). A perfusion-based kidney culture model was developed and optimised The characterisation of rat primary proximal tubular cells and the cell line NRK-52E, as compared to rat kidney *in vivo*, was conducted by using gene expression profiling). Close proximity indirect filter-based renal co-culture systems with epithelial/endothelial and epithelial/fibroblast partner cells were developed .

8. A wide number of cytomic assays (including matrix metalloproteinase secretion, tissue inhibitor of matrix metalloproteinase secretion, IL-6 secretion, RANTES secretion (P5), ATP production, inner mitochondrial membrane potential (JC-1), ROS production, LDH release, lactate production, DNA synthesis and resazurin reduction) have been examined for their usefulness for *in vitro* toxicity testing. Three assays (LDH release for cytotoxicity, BrdU incorporation for DNA synthesis and resazurin reduction related to viable cell number) were selected, based on their ease of application, global toxicological relevance and sensitivity, which will be utilised to identify sub-lethal concentrations of the test compounds. Cytomic data were generated from HK-2 cells intoxicated with ochratoxin A, cyclosporine A, FK506, rapamycin and cadmium chloride .
9. Gene array experiments were conducted comparing plastic grown HK-2 cells, filter grown HK-2 cells in monoculture and HK-2 cells cultured in co-culture with microvascular endothelial cells . Gene expression profile experiments for ochratoxin A (OTA) were conducted using HK-2 cells, human primary proximal tubular cells and rat primary proximal tubular cells. Comparison of different renal culture models was completed and a consensus reached as to the model of choice for further testing and toxicogenomic profiling. This model was HK-2 cells cultured on plastic supports in monoculture.
10. Identification of altered gene expression, generated following exposure to CsA, FK506 and rapamycin (P6) and ochratoxin A. Gene expression profile experiments for CsA, CdCl2, diquat dibromide, FK506, and rapamycin were conducted using the HK-2 cell model. A number of genes were identified that are considered to be potentially related to the cell injury process triggered by nephrotoxin exposure. This list will be expanded once the data for all nephrotoxins has been completed and statistically analysed.
11. Preliminary proteomic analysis using CsA as a test compound, has revealed a number of differentially expressed proteins that could serve as potential markers.

Major publications:

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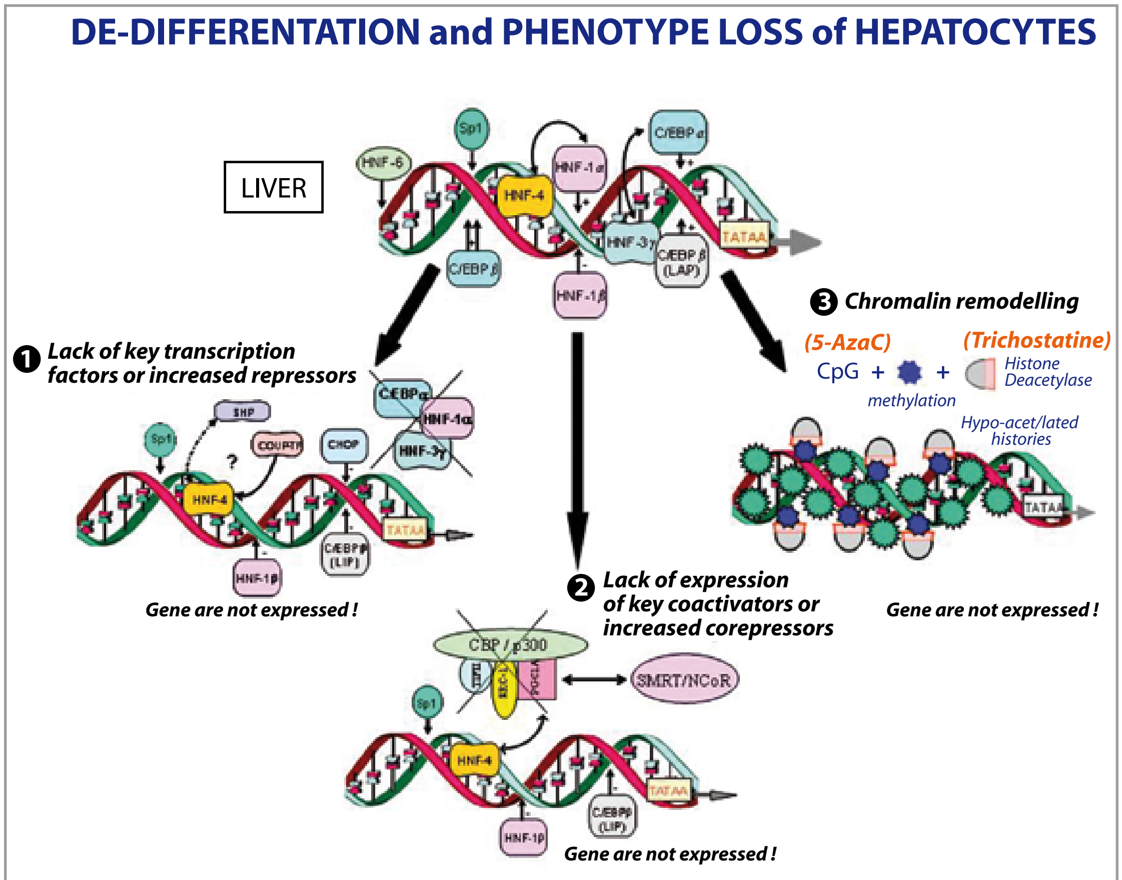

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ReProTect

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 No	**LSHB-CT-2004-503257**
Project type	**Integrated Project**
EC contribution	**€ 9 100 000**
Starting date	**1 July 2004**
Duration	**60 months**
Website	**www.reprotect.eu**

Background and objectives:

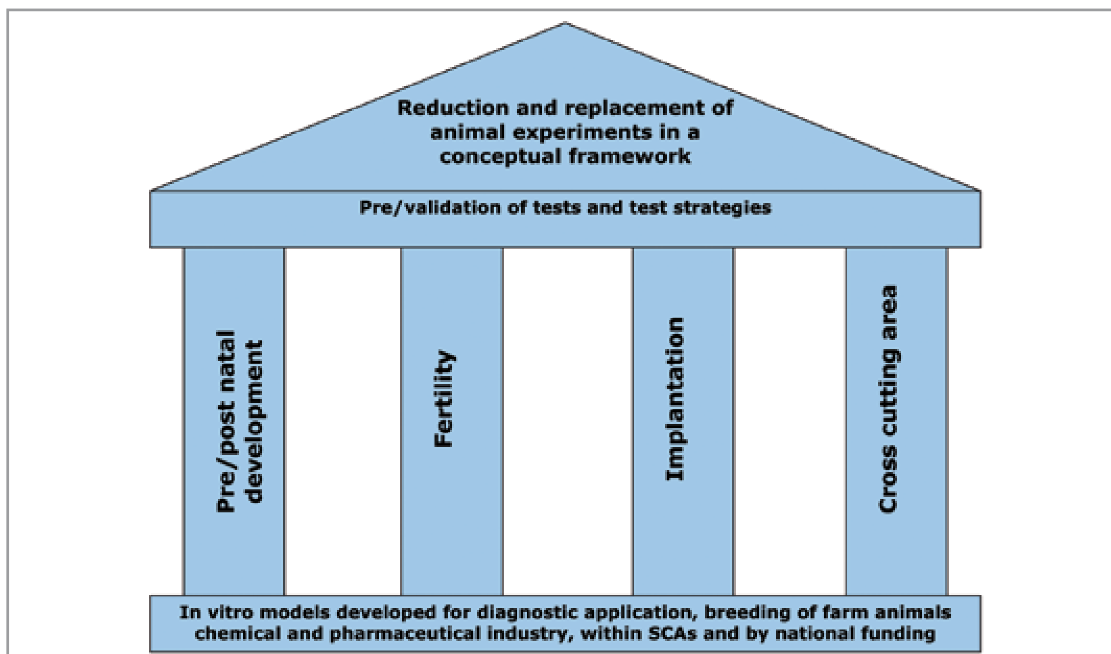
The development of reliable testing strategies for industrial chemicals, biocides, pesticides and cosmetic ingredients is not only relevant for reducing the cost of testing and animal experimentation, but it also serves as a sound basis for the adequate risk assessment of chemicals.

It has been estimated that the detection of reproductive toxicants under the Registration, Evaluation and Authorisation of Chemicals (REACH) initiative will have a very great impact on the use of animals for regulatory safety testing. Under the new legislation, it is estimated that around 5 000 chemicals will require testing for reproductive toxicity and developmental toxicity. By following the present regulatory safety testing guidelines, millions of animals will be necessary just to detect reproductive toxicants for the evaluation of existing chemicals in Europe. In addition to the huge number of animals required by the exercise, the costs are very high. The testing costs for the development toxicity study and for the two-generation study will represent up to 60% of the total testing costs of REACH.

In order to reduce the number of animals, as well as the testing costs, we need intelligent testing strategies which both make use of existing information and also integrate alternative information sources such as *in vitro* tests, QSARs and read-across approaches. In certain areas of reproductive toxicity testing, a number of useful and promising *in vitro* models are already available, but they need to be converted into tests with predictive power for toxicological safety testing. ReProTect is aiming to optimise these tests in order to prepare them for formal validation studies. *In vitro* test batteries will provide a more detailed understanding of the main chemical target tissues or targeted biological mechanisms in the reproductive cycle, such as gametogenesis, steroidogenesis, embryogenesis, etc., and will thereby support regulatory decisions.

Approach and methodology:

The reproductive cycle can be broken down into fundamental elements, as shown in the Work Packages structures of ReProTect. However, due to its pioneering function and its strategic components, the project's role lies far beyond the field of reproductive toxicity. For the first time, complex tiered test strategies will be devised and integrated in a conceptual framework for safety assessment. This entirely new approach for hazard and risk assessment will have to be fully evaluated as to its relevance and reliability. This framework is to be expanded during a a series of consensus workshops. ReProTect is based on several innovative technologies in order to develop the test strategy (e.g. genetically engineered cells and reporter genes).
In the area of prenatal development, murine and human embryonic stem cells will be used. The establishment of human embryonic stem cells in 1998 raised hopes in many research areas, in-



▲ Structure of the project

cluding the development of alternatives to animal experiments. ReProTect is using human embryonic stem cell lines for embryotoxicity testing *in vitro*, which is a unique approach.

Furthermore, some of the toxicological endpoints are based on novel technologies, including proteomics, genomics and sensor technologies. The systematic analysis of these techniques in a defined toxicological area will help to estimate the value of these new technologies. However, the main innovation of the project remains the development and prevalidation of toxicological test strategies based on *in vitro* and *in silico* methods, and their integration into a conceptual framework.

Expected outcome:

Several toxicological endpoints that can assess the hazards of chemicals to male and female fertility will be covered by *in vitro* tests included in the 'Fertility' research area. A reliable test battery for testicular toxicity, which explores various aspects, such as sperm motility, sperm DNA damage and toxicity on Sertoli and Leydig cells, is expected soon. Moreover, the toxicity of chemicals on female fertility is assessed using a multiparametric test on folliculogenesis and a preliminary series of tests for female germ cell toxicity. Optimised *in vitro* tests are now available, and formal validation of the established tests is expected after an independent statistical evaluation on the data is produced.

In July 2006, a new research area emerged, following the selection of new project participants. Research area 2, called 'Implantation' aims to establish cell culture models and explants specifically for the creation of the endometrium, and to identify *in vitro* models for the assessment of placental toxicity. The development of *in vitro* tests based on human endometrial endothelial and stromal cells, as well as models using explants tissue, is expected. Furthermore, the feasibility of cell and tissue culture models specific for assessing adverse effects on the process of placentation, is foreseen.

Moreover, further development of the murine embryonic stem cell tests (EST — already validated for detecting chemical effects during cardiac cell differentiation) is to be expected. New endpoints able to assess effects on neural and skeletal tissues, as well as a metabolic system, will be included.

In order to avoid interspecies variations in drug testing, the adaptation of the murine system to human embryonic stem cells is currently being evaluated and is expected soon. Furthermore, ultra-sensitive, differential protein expression analysis of differentiating mouse and human ES cell cultures exposed to embryotoxic drugs and other chemicals is expected to identify specific protein expression profiles.

Research area 4, 'Cross-cutting', has been established in order to support the other three research areas. QSAR tests on barrier functions (placenta/ blood/ testis), as well as QSARs tests on metabolic systems are already developed and promising results are now available. In this research area, the development of *in vitro* methods for detecting endocrine disrupting (ED) function of chemicals is anticipated. Advanced test methods (PALM, MELN, ER-CALUX, AR-CALUX) and receptor binding assays are now optimised, and the assessment of the transferability of the models is in place. The receptor binding optimisation is also being conducted under the umbrella of OECD as part of the international collaboration with the USA and Japan. Due to the advanced stage of these methods, an *in vitro* testing battery for ED is expected in the near future.

Main findings:

During the first two years, ReProTect explored the predictive power of a range of pioneering *in vitro* tests. It is envisaged that all tests will provide information on the mechanistic basis of a standard operation procedure (SOP), and an assessment of the intra-laboratory reproducibility of the test, according to the ECVAM Modular Approach of validation. More than 150 peer-reviewed reproductive toxicants with different toxicological mechanisms have been selected, in order to support the optimisation process of test protocol development.

ReProTect has successfully identified, developed and optimised 11 tests that could be incorporated into a reproductive toxicity testing battery. These include mechanistic endpoints directed at effects on leydig/sertoli cells, folliculogenesis, germ cell maturation, the motility of sperm cells, steroidogenesis, fertilisation, and the embryo preimplantation.

ReProTect is the first project that explores the potential of human embryonic stem cell (hES) research upon neuronal systems, and builds upon murine (mES) research on differentiation, including neural, cardiac and skeletal cells. Various approaches, such as the identification of protein biomarker signatures, as well as the monitoring of selected markers by using histochemical and molecular biological parameters after exposure with teratogenic compounds, are being followed. A robust SOP for the maintenance of the hES cells and a preliminary protocol for neuronal differentiation is now available within the project. To ascertain inter-species variability, a comparative assessment of hES and mES is being conducted.

In addition, five new partners have been recruited in order to analyse toxic effects upon implantation. This research area is divided into two Work Packages, one concerning the regular preparation of the uterus for implantation as a target for chemical insult, the other addressing placental functions. Several *ex vivo* models and *in vitro* models, such as chorionic villi explants, trophoblast cells, pericyte/ endothelial primary cultures, as well as the placental perfusion system, will be developed in the next two years. With respect to ED hazard assessment, six tests for screening for putative (anti) estrogenic and (anti) androgenic compounds have been optimised, and are now being analysed for their predictive power.

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Sens-it-iv

Novel testing strategies for *in vitro* assessment of allergens

Contract No	LSHB-CT-2006-018681
Project type	Integrated Project
EC contribution	€ 10 999 700
Starting date	1 October 2005
Duration	60 months
Website	www.sens-it-iv.eu

Background and objectives:

With some forms of allergy on the rise, massive resources are being invested worldwide to investigate which compounds are the culprits and why some otherwise harmless compounds do elicit adverse immune responses. The need for non-animal alternatives is now all the more important because of existing and pending EU regulations. The 7th Amendment to the Cosmetics Directive (Directive 76/768/EEC) will completely ban all animal testing for cosmetic ingredients by 2009 at the latest. The ban covers skin sensitisation. Conversely, the new EU legislation on chemicals (REACH) will require a great deal of additional chemical testing. It is estimated that skin sensitisation testing is among those human health effects that require large numbers of animals.

Therefore, 28 groups from academia and industry, as well as special interest organisations, have joined the Sens-it-iv consortium to develop non-animal tests and testing strategies to assess allergenic potential. The overall goal of Sens-it-iv is to develop strategies to replace animal experimentation by *in vitro* assays for identifying skin and respiratory sensitisers. This is seen in relation to the use of safe ingredients by the chemical, cosmetic and pharmaceutical industry.

Approach and methodology:

Sens-it-iv will build on and exploit our increasing understanding of the mechanisms through which chemicals and proteins induce allergy to develop novel, realistic and accurate methods for safety assessment and product development. Sens-it-iv aims to acquire a solid understanding of the processes occurring *in vivo* when tissue is challenged by a potential sensitiser, and to compare this with molecular indicators on the cells involved in these reactions. Based on this understanding, the partners will develop assay systems that model sensitisation, rather than irritation and toxicity of chemicals and proteins.

Scientific objectives

The scientific objectives of Sens-it-iv are as follows.

- Use of functional genomics, proteomics, and immunohistology before and after the chemical challenge of tissue slices, so as to address *ex vivo* phenotypic characteristic changes of human epithelial cells (EC), antigen presenting dendritic cells (DC) and effector T cells.
- Establishment of *in vitro* conditions supporting an *in vivo*-like crosstalk between EC, DC and T cells, and the cascade of cellular and molecular events triggered in such a complex system by a test-compound.
- Description of the chemical features related to the intrinsic stability of allergens in relation to the metabolic capacity of cells in the target tissue. Chemical structures and peptide sequences involved in hapten formation will be characterised.

Sens-it-iv

The processes of bio-activation and hapten formation will be studied by advanced metabonomic and proteomic technology.

Technological objectives

Sens-it-iv has the following technological objectives:

- set-up of an inductive database for the acquired scientific data, including all available literature information to allow queries for data patterns and predictive models;
- creation of prototypes of cell-based predictive assays through the implementation of the cellular and excreted markers suggested by bio-informatics to represent key mechanisms of sensitisation;
- refinement and optimisation of these assays for prevalidation.

Expected outcome:

The coordinated and extensive characterisation of the impact of compounds on cellular-molecular interactions will identify key mechanisms of sensitisation. The development of a technology platform will allow queries for the following:

- data, regularities, patterns and models within the data;
- predictive models, and new methods and strategies for the design of safer chemicals and drugs.

The ultimate goal of Sens-it-iv is the establishment of *in vitro* assays that are ready for prevalidation and approval by ECVAM, the European Centre for Validation of Alternative Methods.

Main findings:

In its first 12 months , the Sens-it-iv project made significant progress towards its goals:

- A learning panel of 9 chemicals (including 3 skin sensitisers, 3 respiratory sensitisers and 3 controls) and 2 proteins was identified, stored and distributed from identical batches to all participants
- Precision Cut Lung Slices (PCLS) technology for the testing of lung sensitisers was refined, resulting in a standard operation procedure for *ex vivo* profiles of lung epithelial cells (EC), antigen presenting dendritic cells (DC) and effector T cells.
- A variety of human cell lines representing the major players in allergy is being studied in a coordinative effort, with standardised culture- and test-conditions already emerging.
- A unique catalogue of gene expression profiles for EC and DC of different origins was established. The transcriptome responses by A549 alveolar cells to proteases were analysed, revealing a number of new markers and pathways of potential interest to this project.
- An epidermal equivalent model with normalised expression of defined *in vivo* markers was exposed in pilot experiments to the learning set of chemicals.
- Protocols for generating distinct allergen-specific effector/memory T cells, for performance of DC – T cell co-cultures, were established. The development of a sustainable adaptive immune response model for assessing the allergenic potential of compounds was initiated. Various *in vitro* T cell priming assays were assessed, and a new protocol of polyclonal 'cell amplification' was established.
- Proteome expression profiles of primary human keratinocytes and human HaCat cells were analysed by two-dimensional electrophoresis and preliminary differential experiments with selected compounds, and are now ready for comparison.
- Hapten formation as a metabolic route was addressed using radiochemical and immunochemical methods for detection of protein adducts in order to elaborate

pathways of bio-activation and bio-inactivation. Emphasis was placed on the use of a cutaneous cytochrome P 450 cocktail to mimic cutaneous oxidative metabolism;

- A central repository for Sens-it-iv data has been created. The basic infrastructure includes the following:
  (i) a version control system, that tracks the progress of the project and can restore previous stages of the project;
  (ii) multiple redundant backup systems to guarantee the integrity of the submitted data;
  (iii) a web interface for the submission and retrieval of Sens-it-iv data.

A monthly newsletter was established on the Sens-it-iv web site to inform the public of upcoming activities involving the project, and recent major breakthroughs.

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TOXDROP

Innovative 'cell on chip' technology to screen chemicals for toxicity, using cultured cells within tiny 'nanodrops' of culture fluid

Contract No	SP22-CT-2004-513698
Project type	Specific Targeted Research Project
EC contribution	€ 1 615 887.89
Starting date	1 January 2005
Duration	24 months
Website	http://toxdrop.vitamib.com

Background and objectives:

The TOXDROP project set out to develop low-cost, rapid and unambiguous toxicological assay screening while respecting the Good Cell Culture Practice.

Traditionally, *in vitro* toxicology assays are performed in Petri dishes or microtiter plates with over 10 000 cells per well. This amount of cells is inadequate if each cell needs to be wholly phenotyped. To streamline the manipulation of microtiter plates, and to reduce the number of cells per assay, the TOXDROP project partners recommended using the alternative method 'DropChip', where cells are cultured in nanodroplets on a microscope glass slide. Using this method, the partners anticipated carrying out multiparametric assays and significantly increasing the accuracy in measuring the cell response to toxic addition.

TOXDROP, which was carried out over a period of 2 years, allowed successful *in vitro* toxicological studies using nanodrop cultures, and placed this innovative concept in the context of the development of 'cell on chip' devices.

Approach and methodology:

The drops were maintained at a defined position throughout the experiments using a hydrophilic/hydrophobic interface which enabled stabilisation and individualisation of the drops for high-throughput capabilities. The culture conditions on chip were set so as to obtain approximately 100 cells at fixation time (day 4) in the non-exposed cultures, which was enough to provide statistical analysis significance while avoiding growth inhibition and stress due to excessive cell density. In order to develop a proactive strategy and record the toxicology of various chemicals using 'cell on chip', the partners used this reduced number of 100 cells per drop assay and realised a high level of cell behaviour characterisation.

Using the metallic mesh embedded in the chip for precise positioning, TOXDROP performed automated smart capture using IMSTAR's (partner 5) Pathfinder™ system. The Pathfinder™ imaging platform enables a fully automated capture of the whole chip with intelligent image-data management (IDB, patent n° 01921459.2) for each spot at 0.6μm resolution compatible with multispectral fluorescence single cell detection and multiparametric cellular characterisation. Its multiplex capability makes the platform a good tool for complex phenotyping in large scale toxicology.

The partners also set up culture conditions and protocols for cell viability and differentiation of hepatotoxicity for a new human hepatoma cell line HepaRG in drop culture. A new specific fluorescent oxidative stress assay based on EGFP reporter gene induction was engineered in HepaRG cells under the control of the hsp22 promoter; it allows a selective induction of the construct restricted to differentiated hepatocyte colonies and to oxidative stress, especially heavy metal related.

TOXDROP

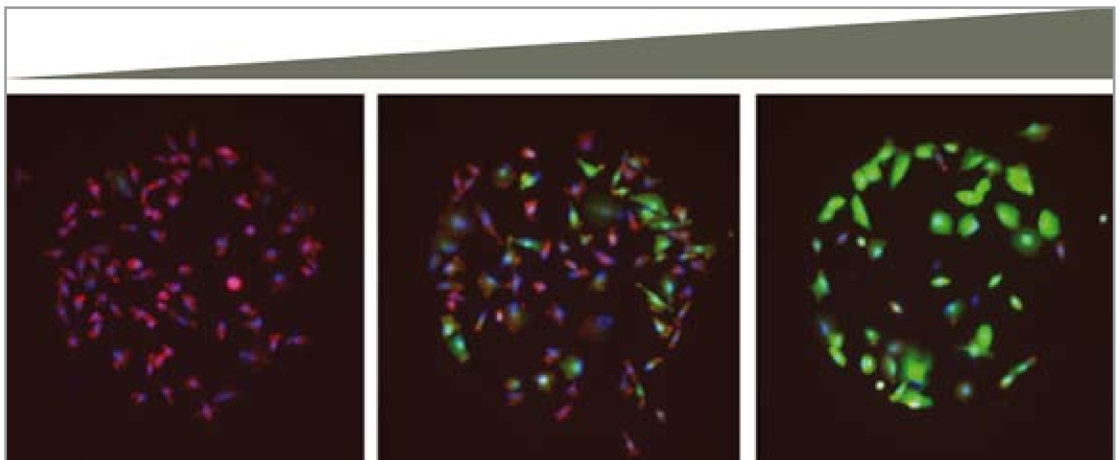
Main findings:

TOXDROP aimed at providing an original approach for automated High-Throughput High-Content Screening in toxicity assays using a revolutionary format of cellular nanodrops formed on a glass slide.
Two individual approaches were tested in order to facilitate toxicity analysis within these cellular nanodrops: on the one hand, fluorescence microscopy was used to identify fluorescent proteins (fluorimetric assay), while on the other hand, 2- and 3-dimensional mass spectrometric analysis of cells was performed (ToF-SIMS analysis). New software tools were developed for the analysis of the images provided by both approaches.

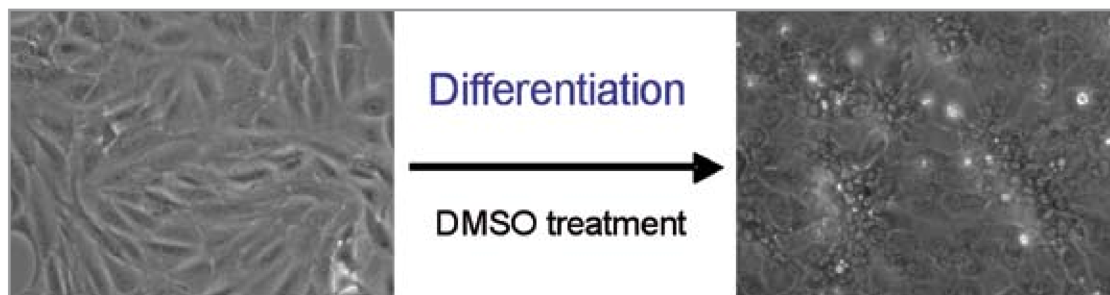
Nanodrops are realised through conventional piezo dispensing or by using a microfluidic device (CellJet), developed through collaboration between partners 1 and 4 (CEA and EPFL). This device is based on impedance detection of cells within the chip and allows ejection of one cell per drop to obtain 100 cells containing drop-assays. Additionally, specific substrates for the Nanodrops were engineered.

TOXDROP developed a fluorometric cell-based assay for global toxicity monitoring using the "stress promoter technology". For that purpose, several cell lines have been generated by partner 3 (UCBL). They were chosen as models of organs that are particularly sensitive to toxic chemicals, such as liver cells (HepG2), lung bronchial epithelium cells (16HBE), keratynocytes (HaCat) and differentiated hepatocytes (HepaRG, in collaboration with partner 2, INSERM), and which could express Enhanced Green Fluorescent Protein (EGFP) upon induction of heat shock family stress promoters . Such engineered cell lines are able to respond to the presence of a chemical stress by increasing the expression of the fluorescent reporter protein EGFP (Figure 1).

Figure 1: EGFP reporter gene expression is induced in the presence of increasing concentration of toxic chemicals. The Hsp induction is monitored by the green EGFP signal, cell nucleus is stained in blue by Hoechst and actin cell cytoplasm is stained in red by Phalloïdin.



One key development of the "stress promoter technology" is the development of Hepatocytes HepaRG cells. This cell type displays active biotransformation capacities and was transfected by a specific vector by partner 3 (UCBL) and partner 2 (INSERM). Selection of individual clones was performed 3 days after transfection and 4 days

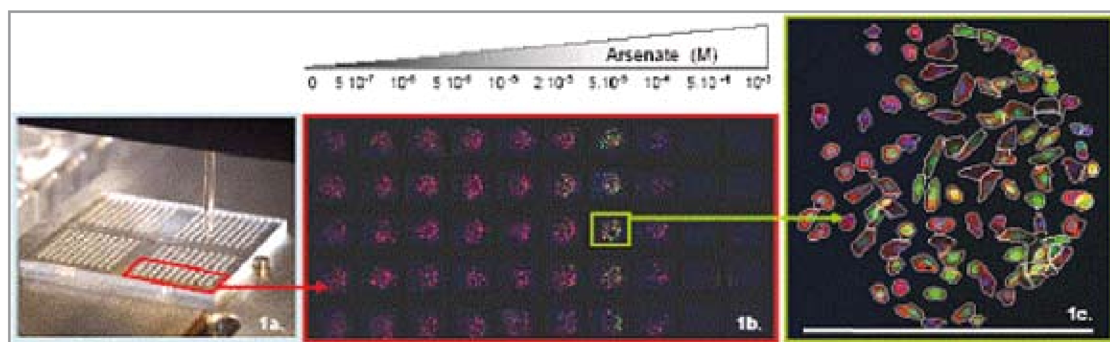


after DMSO treatment. It allows a selective induction of the construct by oxidative stress, which particularly relates to heavy metals, restricted to differentiated hepatocyte colonies.

In order to perform the fluorimetric assay, the engineered cell lines were provided to Partner 1 (CEA) in order to set up dispensing conditions, and to allow their culture on 'cell on chip' devices. The high level of proliferation allowed the network to obtain sufficient cell density to test several chemical stress conditions. A 'cell on chip'

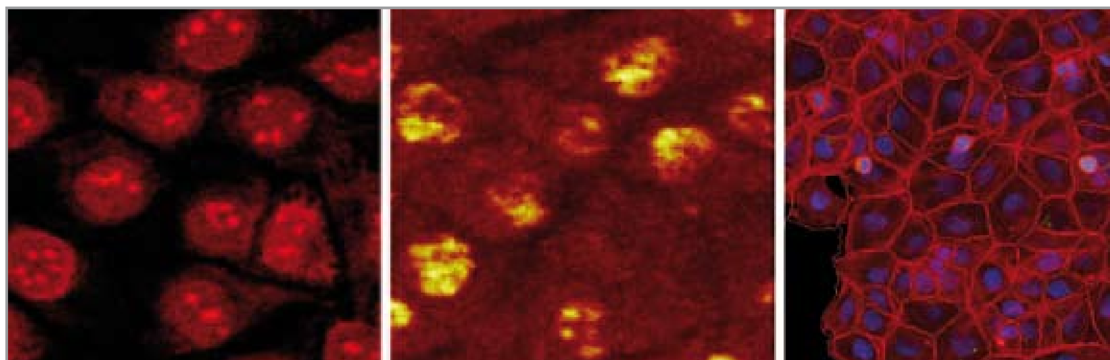
▲ Figure 2: HepaRG. Differentiation appears after 4 days of DMSO treatment.

Cellular analysis and multiplex phenotypic characterisation was performed using IMSTAR (partner 5) Pathfinder™ leading technologies for HCS analysis. Using this patented technology, an automated and quick detection of cell borders, as well as fluorimetric response of cells were possible. Thus, the enormous amount of data obtained by both the fluorimetric, as well as the mass spectrometric approach developed within



device was used to analyse 400 independent nanodrops. Each nanodrop contained 100 individual engineered HepG2 cells and was spiked with an individual species and concentration of a toxic substance. The results obtained showed an increasing fluorescence signal upon increasing the concentration of the toxic substance (figure 3; published in PLoS).

▲ Figure 3: Multiplexed toxicity assay in drops. 2a. Cell dispensing with sciFlexarrayer robot arrayer. 2b. Zoom on the assembled mosaic of images corresponding to HepG2 hsp22-EGFP clone after 6h exposure to 10 doses of arsenate in quintuplicate measurements (columns); the Hsp induction is monitored by the green EGFP signal, cell nucleus is stained in blue by Hoechst and cell cytoplasm is stained in red by Phalloïdin. 2c. Heterogeneity in cell response is illustrated by an example of Hsp response to 5.10-5M arsenate exposure. Scale bar represents 500 µm. Fully automated image capture with a 10× objective and dedicated image analysis were performed using the same detection protocols by IMSTAR Pathfinder™ Cellscan system.



▲ Figure 4: ToF-SIMS assay and application of Pathfinder™ technology: Detection of a fluorophor on an identical sample position by fluorescence microscopy (left) and ToF-SIMS Imaging (middle). Cell border detection by IMSTAR Pathfinder™ technology on a fluorimetric assay nanodrop (right).

the TOXDROP project, could be evaluated. Given the large amount of information to be processed, a shared database was initiated to facilitate data storage and implementation of statistical tools.

Pathfinder™ technology created a bridge between these two approaches in order to finally provide a comprehensive platform integrating all characterisation data with full compatibility and transparency for the user, although the physical nature of image fluorimetry and mass spectrometry is very different. It is to highlight that IMSTAR developed integration software to make the Pathfinder™ technology capable of reading Tascon mass spectrometry data and to organise them within the Pathfinder™ database.

Major publications:

Mandon, C.A., Diaz, C., Arrigo, A.P., Blum, L.J., 'Chemical stress sensitive luminescent human cells: molecular biology approach using inducible Drosophila melanogaster hsp22 promoter', *Biochem Biophys Res Commun* , 2005, 335(2):536-44.

Mandon, C.A., Diaz-Latoud, C., Arrigo, A.P., Blum, L.J., 'Dithiocarbamate fungicide thiram detection: Comparison of bioluminescent and fluorescent whole-cell bioassays based on hsp22 stress promoter induction', *J Biotechnol*, 2006, Jul 13;124(2):392-402. 2006.

Lemaire, F., Mandon, C.A., Reboud, J., Papine, A., Angulo, J., Pointu, H., Diaz-Latoud, C., Lajaunie, C., Chatelain, F., Arrigo, A.P., Schaack, B., 'Toxicity assays in nanodrops combining bioassay and morphometric endpoints', *PLoS ONE*, 2007, Jan 17;2:e163.

Cerec, V., Glaise, D., Garnier, D., Morosan, S., Turlin, B., Drenou, B., Gripon, P., Kremsdorf, D., Guguen-Guillouzo, C., Corlu, A.,[2] 'Transdifferentiation of hepatocyte-like cells from the HepaRG cell line through bipotent progenitor', *Hepatology*, 2007 Apr;45(4):957-67.

Patent:

A new HepaRG cell line carrying a marker of hepatic oxidative stress French and English.

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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 No	**LSHB-CT-2006-018940**
Project type	**Specific Targeted Research Project**
EC contribution	**€ 2 942 000**
Starting date	**1 January 2006**
Duration	**36 months**
Website	**http://er-projects.gf.liu.se/~vitrocellomics**

Background and objectives:

The objective of VITROCELLOMICS is to establish stable cell lines that reliably reflect human hepatic properties through the development of models derived from human embryonic stem cells. The aim is to deliver reliable *in vitro* models that could be used by the pharmaceutical industry to replace experimental animals in investigations on human drug metabolism, uptake and efflux properties of compounds in the drug discovery, and development processes. In the pharmaceutical industry, reliable *in vitro* cell models would replace current techniques and animal experimentation in the selection and optimisation of lead compounds, as well as in the documentation of a selected drug candidate before it enters clinical development.

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 failed in the late clinical phases because of pharmacokinetic problems. Moreover, adverse drug reactions, most of which are pharmacokinetic-based, are the 4th-6th 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, in order to be able to select high quality compounds that could be developed into drugs that are safe and easy to administer.

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 that are available. Consequently, 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 the documentation of a selected drug candidate before it enters clinical development. *In vitro* cell models that could reliably predict human metabolism and disposition would reduce the need for animal experimentation for this purpose by 60-80%.

Approach and methodology:

The means to accomplish the objectives of VITROCELLOMICS are, in addition to new stable human embryonic stem (hES) cell derived hepatocytes, the following:

- 3D-hepatic cell culture and co-culture methods;
- microcultivation monitoring systems for *in vitro* screening;
- genomic and metabolomic characterisation;

- a multi-micro-bioreactor platform for high-throughput screening of drug candidates.

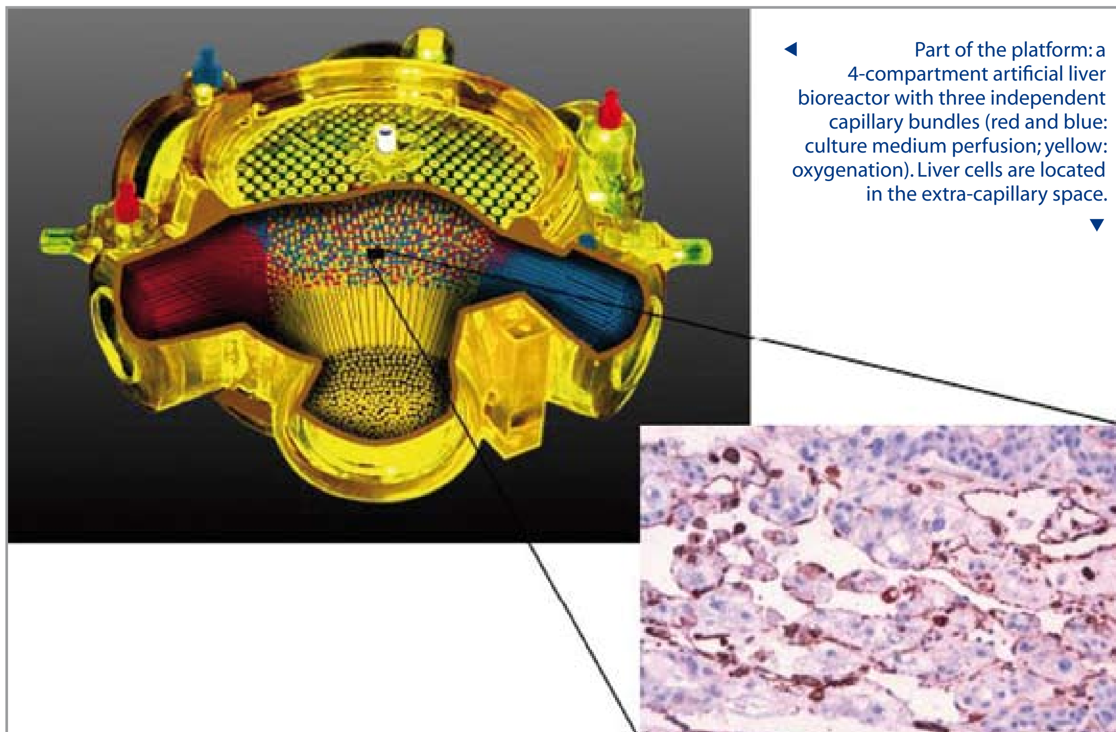
Comparative studies of hepatocytes derived from hES cells with established *in vitro* models will be carried out in order to validate the new models and methods.

The overall objectives of VITROCELLOMICS are:

- replacing animals in preclinical pharmaceutical development by human cell 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 to enter clinical phases with innovative human hepatic cell systems;
- delivering an *in vitro* testing system with adjacent methodology pertinent for validation in GLP/SOPs environment for absorption, metabolism, and toxicity;
- reducing, or even totally abolishing, the use of animals in drug testing, refining the model system under consideration, and replacing the animals currently used, which is the ultimate aim.

As measurable and verifiable specific objectives for the project, the following 4 technology-related objectives are defined:

1. establishing relevant hES cell derived hepatocyte cultures that allow a more predictable preclinical lead testing programme to be carried out;
2. developing a 3-dimensional *in vitro* model for long-term studies of drug metabolism and toxicology that mimics the *in vivo* tissue or organ cyto-architecture and function;
3. establishing a versatile cell lab platform



Part of the platform: a 4-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.

based on the developed cell lines and cultivated in advanced miniaturised bioreactor systems with non-invasive measurement techniques for *in vitro* testing of metabolism, toxicology and absorption;
4. quantifying metabolism by *in vitro* assay methods.

Expected outcome:

The expected outcome of the project is new, efficient *in vitro* prevalidation models, which will significantly reduce the use of animal experimentation for prediction of human drug metabolism by 60-80%. In addition, the models will also increase safety and quality of compounds chosen as candidates in the different stages of the drug discovery and development process. Furthermore, it will strengthen the possibility for SMEs to market new potential products in the areas of cell assays and *in vitro* compound screening.

The clinical expertise in the project is manifested by the involvement of 2 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. Also, the coordinator has managerial background from an international pharmaceutical company, while the project leader is from one of the SME partners. Three partners represent a solid reputable bioengineering background. Other important partners are one SME, which is focused on the 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 comprised in the project.

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BBMO

Biosensors based on membrane organisation to replace animal testing

B iosensors
B ased on
Molecular
O rganization

Contract No	LSSB-CT-2005-005199
Project type	Specific Support Action
EC contribution	€ 443 878
Starting date	1 January 2005
Duration	30 months
Website	www.chem.umu.se/bbmo

Background and objectives:

The ultimate goal of the BBMO project is the development and validation of alternative techniques to replace animal testing in drug screening and environmental control protocols. This Specific Support Action was organised as the first step towards uniting fragmented industrial and academic research groups to form a powerful platform in biophysical chemistry and biosensor technology. Since the programme's launch, smaller working and expert groups have focused on the study of relevant research areas. The results of this work were discussed at a Strategic Meeting during the course of the project.

- The first objective was to initiate and carry out five illustration projects within the project's timeframe, in order to facilitate the integration between the research groups in the consortium.
- The second objective was the organisation of a Strategic Meeting focusing on particular tasks: synthesis, film formation and characterisation, and modification with functional molecules.
- The third objective is to finalise a prospective analysis that supports future developments in membrane-based biosensing technology, validated by comprehensive inter-calibration techniques.
- The fourth objective is to produce a Work Programme consisting of a set of Work Packages (WPs).

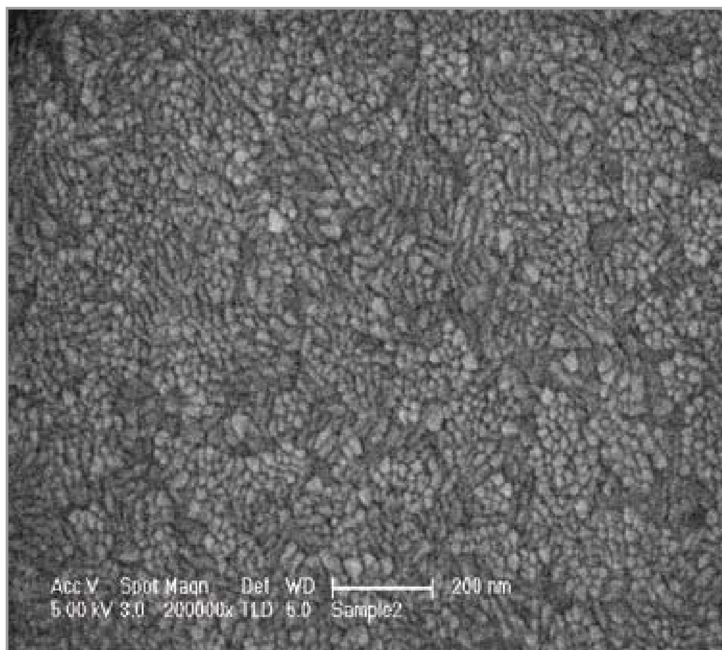
Approach and methodology:

A kick-off meeting was arranged in Sweden at the very beginning of the project, where all the partners met and presented their own competences, thoroughly discussing how each respective partner could contribute to the project. It was possible to identify five smaller clusters of people in good accordance with what was outlined in the original proposal. Furthermore, an intense discussion through mailing and web-conferences followed, which resulted in the following five illustration projects that have been running throughout the project, where several partners are involved.

1. Langmuir Blodgett films on cellulose acetate.
2. Investigations of perfluorooctane sulfonate (pfos), perfluorooctanoic acid, (pfoa) and fluorotelomer alcohol at lipid membrane systems.
3. Peptide interaction with lipid layers.
4. Hydrophilic repellent films on gold.
5. Development of a glucolipid membrane surfaces from achyloplasma bacteria.

The main idea was to find ways to integrate research between the various laboratories with the small resources that were at hand, so that the goals could be met. Several papers have been submitted for publication or are in manuscript. Three web courses were planned during the project and ran with modest participation of some of the partners or their PhD students.

BBMO



◄ Scanning electron microscopy image of a LB monolayer of cellulose membrane deposited on a solid support (indium tinoxide). Result of a collaboration project between Prof. Mandler, HUJI, Jerusalem and Prof. Vadgama, QM London.

Full paper in preparation from electrochemical characterisation of the biomimetic film

As this is not a research project, the approach to these courses is introductory and not of examination character. The three web courses are as follows.

- 'Electrochemical Impedance with focus on Lipid systems.' A podcasting course was prepared by Britta-Lindholm-Sethson at UmU. During this course, some of the experimental outcomes from the illustration project were included.
- 'Evince web course' run by Umbio AB and made available on their homepage in 2005.

'Example of Fourier Transform in Electrochemistry' run by Magnus Rosvall at Rosvall Instruments.

Overall, several new and unique research projects have been initiated throughout the project period, although the project did not have any support for research. The main reason behind the success is the great enthusiasm among the many partners that brought in PhD students and post docs in the illustration projects, but also the coordinator's efforts, travelling to all partners, e-mailing and arranging web-conferencing.

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CONAM

Consensus networking on alternatives within Europe

Contract No	**LSSB-CT-2004-504776**
Project type	**Specific Support Action**
EC contribution	**€ 150 000**
Starting date	**1 March 2004**
Duration	**36 months**
Website	**www.ecopa.eu**

Background and 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 3R-alternatives, ideally including all European countries (existing and new EU countries, as well as candidate countries), and with links to 3R-relevant organisations and institutes. One partner, VUB/ecopa, was involved in this project. Ecopa groups 16 national consensus platforms, of which 13 are full members and 3 are associates.

Expected outcome:

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. Thanks to this contact building, Hungary and Poland established the NCPs hucopa and polcopa, respectively.

Already existing organisations adapted their structure according to the ecopa rules, so as to have representatives from the four parties concerned, e.g. Germany with the Stiftung set, and the UK with the Boyd group. New platforms were also launched, in Norway, Denmark and Ireland, for example, resulting in a current total number of 16 NCPs (Belgium, the Czech Republic, Denmark, Germany, Ireland, Spain, Italy, Hungary, the Netherlands, Austria, Poland, Finland, Sweden, UK, Switzerland and Norway). France is expected to join in 2007, and is now in the phase of building a NCP according to ecopa's rules.

Main findings:

CONAM has succeeded in doing the following:

- The ecopa website was improved, expanded from a simple webpage to a full website, complete with news and events sections, databases and links, an archives section, information pages about education and legislation, and a forum.
- The ecopa newsletter was created: 10 issues of the 'ecopa messenger' have been sent out to date, and they are also available on the ecopa website.

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.

An ethics working group was formed; it organised a consensus training course in Ljubljana in June 2005. The report is available on the website. A workshop on 'The Use of Human Tissue' was organised in Brussels in November 2006 and the report is made available on the project website.

An educational workgroup was created and the Spanish platform REMA, being part of it, organised 2 training courses on alternative methodologies in 2006. A similar initiative was taken on behalf of the Italian platform, IPAM, in Rome in February 2007.

A workgroup on 'Chemical Policy, REACH' was created. Besides the organisation of several workshops, (e.g. in Brussels in November 2006, where discussions focused on the limitations and problems of REACH as regards the high numbers of animal use, and the implementation problems of alternatives), the workgroup also prepared a report on the impact of REACH on animal use.

During the run of CONAM, several workshops were organised.

- On an 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.
- Within CONAM, the workshops 'ecopa Science Initiative (eSI)' were organised on 2 occasions in Pueblo Acantilado in Spain, in October 2004 and September 2006. The idea to organise these workshops was developed during the annual ecopa workshop in November 2003: ecopa had performed a literature analysis to find out how many original research ideas in the field of the 3Rs were developed within the last five years. It was concluded that the field had an urgent need of new and fresh initiatives. Therefore, young and promising scientists were brought in 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.

In order to stay updated with respect to developing research within the 3Rs, the workgroup on '3Rs-research' was created within the CONAM project. 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.

Within the CONAM project, contact building with relevant bodies and third parties has been realised, e.g. with IVTIP, OECD, Colipa, CEFIC, ESAC, SusChem and the epaa initiative, among others.

Also running is an EU study, 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. This will provide a comprehensive picture of EU research in this area, and will be of great assistance in planning an EU-coordinated research programme. 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 the ecopa website (available since 1 February 2006).

CONAM

Major publications:

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.

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

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.

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

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ForInViTox

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

Contract No	**LSSB-CT-2007-037779**
Project type	**Specific Support Action**
EC contribution	**€ 288 850**
Starting date	**2007 (date to be confirmed)**
Duration	**24 months**

Background and 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, 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 establish a forum where representatives of manufacturers, research projects and regulatory agencies continuously get a chance to discuss how to speed up the process of making *in vitro* methods available for end users.

The following activities will take place:

- a retrieval of existing, not properly exploited knowledge;
- an analysis of the needs of toxicity tests;
- an inventory of the producers of *in vitro* toxicity tests.

Approach and methodology:

The project is structured into 6 Work Packages (WPs). Three are devoted to retrieving the relevant information required: availability of inventions (WP1); present and presumably future needs of users (WP2); and ability of currently existing manufacturers (WP3) to meet the required demands. This will be followed by an analysis of the outcome of these exercises, with the aid of experts (WP4). The ultimate goal is to set up a forum where the interested parties can meet (WP5). A general management Work Package is also anticipated (WP6).

Expected outcome:

ForInViTox will summarise the data from these reviews and identify the most urgent testing needs. The project partners will prepare an analysis -- complete with input from a highly qualified expert group. It will include suggestions for stimulating research, technology transfer, development of smaller manufacturers or other steps to further increase the use of *in vitro* tests.

At the end of the project, a forum event, with representatives from the different stakeholders, will take place. An interactive forum website for discussion, information and education around issues concerning implementation and use of alternative toxicity tests, as well as presentations of innovations and manufacturers will be developed. The outcome of the forum will be a

ForInViTox

White book, recommending actions to meet the increased needs for alternative toxicity tests.

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InViToPharma

Workshop on the need of *in vitro* toxicity tests within the pharmaceutical industry

Contract No	**LSSB-CT-2007-037814**
Project type	**Specific Support Action**
EC contribution	**€ 578 000**
Starting date	**2007 (date to be confirmed)**
Duration	**24 months**

Background and objectives:

A workshop where representatives of manufacturers of toxicity tests, researchers developing new *in vitro* pharmaceutical toxicity tests, the European pharmaceutical industry and regulatory authorities participate makes it possible for stakeholders to discuss the supply and demand for *in vitro* pharmaceutical toxicity tests, new and old.

The workshop will also increase business opportunities for the manufacturers of *in vitro* pharmaceutical toxicity tests, often SMEs, as the participants will have ample time to sit down and confer in smaller groups and one-on-one.

A dialogue between the stakeholders within the area of pharmaceutical toxicity testing is crucial for achieving a more general use of alternative toxicity tests. More efficient toxicity tests are also an important factor in making it possible for the European pharmaceutical industry to boost its competitiveness. The increasing amount of substances passing the drug development process needs to be tested with efficient methods as early as possible for new pharmaceuticals. This demands more robust and cost-efficient methods, which are not always available today.

The main objectives of this project are as follows:

- 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 the development of new *in vitro* toxicity tests, high-throughput screening tests, and tests monitoring the toxicological mechanism in detail, etc.;
- to identify and present academic model systems that are suitable for use in 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 and methodology:

The InViToPharma project will start with a general analysis of the current situation: This is Work Package 1 (WP1) and it will precede a planning meeting with the Advisory committee, involving representatives from the stakeholders. The information that will form the basis for the analysis will be retrieved from questionnaires presented to pharmaceutical companies. The outcome of the compiled answers and a general analysis of the tendencies will be discussed at the second Advisory committee meeting, and will form the base for the workshop programme.

A two-day workshop on the subject 'What is the need of *in vitro* toxicity tests within the pharmaceutical industry' will take place at Silverdal Science Park in Stockholm, Sweden. The workshop will comprise both introductory lectures and

smaller working groups focused on different issues, defined after the general analysis described in WP1. 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.

Expected outcome:

The project will bring together stakeholders within the area of pharmaceutical toxicity testing, researchers in the field of *in vitro* toxicology, the pharmaceutical industry, manufacturers of *in vitro* toxicity tests for pharmaceuticals and regulatory authorities. Drawing together all these players has the aim of attaining an effective interplay, for safer and more efficient and drug development, in line with the 3 Rs principle, within the European pharmaceutical sector.

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NHR DevTox

A prospective analysis of the mechanisms of nuclear hormone receptors and their potential as tools for the assessment of developmental toxicity

Contract No	**LSSB-CT-2004-512513**
Project type	**Specific Support Action**
EC contribution	**€ 144 000**
Starting date	**1 January 2005**
Duration	**11 months**

Background and objectives:

The aim of NHR DevTox was to analyse mechanistic approaches that could be applied for the assessment of developmental toxicity, thus potentially leading to an innovative non-animal testing methodology.

As all normal processes of biological development are ultimately controlled at the molecular level, the NHRDevTox project proposed a unique approach for developmental toxicology by studying the nuclear receptors that are fundamental to these processes. Recent and emerging technical advances in the fields of genomics and proteomics, among others, enable the study of such processes and of the mechanisms underlying their perturbation by exposure to xenobiotics. Accordingly, the prospective analysis focused on the role of nuclear hormone receptors (NHRs) in developmental toxicity.

Approach and methodology:

The methodology proposed for this SSA involved convening two workshops in which a consortium of scientists from leading European research teams active in the area of molecular and cell biology, genomics and developmental toxicology, complemented by external experts, would review the state of the science of NHRs, and their role in developmental and reproductive biology. The options for further study and research were reviewed and discussed, so as to identify the priority areas of research that should be carried out to advance the scientific knowledge of reproductive toxicology, and attain the ultimate goal by highlighting the different technologies needed to deliver success.

The first workshop was designed to review the regulatory background, to describe the state of the science, and to identify the research tools currently available for studying developmental toxicity at the molecular level, with particular reference to the role of NHRs. Discussion was encouraged to highlight the most promising aspects of research that would deliver the objectives set out in the SSA, particularly within the context of evaluating chemical hazards.

The second workshop was designed to develop a cohesive plan of research (with input from invited experts) which would deliver a greatly improved understanding of the role of nuclear hormone receptors in developmental biology, particularly within the context of their perturbation by chemicals causing toxicity. Workshop 2 applied the generic paradigm developed in Workshop 1 to the review and discussion of specific areas considered to be most promising for study.

It is recommended that research is conducted to further elucidate the role of NHR mechanism developmental toxicity, based on the paradigm and schematics designed in this SSA. Such research should consider both upstream and downstream signalling pathways, as well as co-regulators. It

would initially focus upon a particular/specific toxicological endpoint that is capable of being modelled in an animal-based system.

The mechanisms elucidated in such an approach should then be tested for their relevance to humans though the use of *in vitro* systems. If the resultant mechanism of action(s) determined by the approach is relevant to humans, then cell-based systems that are capable of reporting on the ability or otherwise of chemicals to influence those mechanisms should be developed.

Expected outcome:

The major scientific outcome of this work is a generic paradigm on how the developed approaches can be used to determine a mechanism of action of reproductive toxicity, and how they might be applied to future research projects. This is a novel approach for the development of *in vitro* technologies, particularly in that many existing approaches use imperial methods, rather than starting from a mechanistic understanding of the toxicological phenomena for which a test system is intended.

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RAINBOW

Research on animal and *in vitro* studies and numerical methods: bridging opportunities through a workshop

Contract No	LSSB-CT-2005-018695
Project type	Specific Support Action
EC contribution	€ 145 950
Starting date	1 March 2006
Duration	12 months
Website	www.rainbow-project.eu

Background and objectives:

The RAINBOW project organised the RAINBOW workshop, held in Milan, Italy from 11 to 13 December 2006, which addressed the integration of *in vivo*, *in vitro* and computer-based methods (*in silico*) for studying the toxic properties of chemicals. The workshop targeted regulators, academic and industrial scientists and stakeholders, including chemical industries, animal welfare experts and public organisations. Around 50 participants from 14 EU and non-EU countries attended the workshop, which helped break down barriers between scientific and societal areas. The participants discussed the implications of the REACH initiative on the integration of methodologies.

There is an urgent need for test systems that can fill the enormous data gap for untested or insufficiently tested substances, as efficiently as possible. The recent approval of the REACH regulation by the European Parliament is an important step towards addressing this societal request. An efficient test strategy must consider the limitations in economic resources and testing capacity. It must also be in line with the aim of reducing the use of animals in toxicological testing. By 2009, the 7th amendment to the Cosmetics Directive will prohibit the use of animals for premarketing toxicity testing for cosmetic products in the EU (Directive 2003/15/EC, Official Journal L66:26-35).

Individual toxicological and ecotoxicological tests can be described in terms of their cost, validity, reliability, and sensitivity. It should be noted that there is no such thing as the perfect test. If researchers had, for all important endpoints, tests that fulfil the criteria of low cost, sufficient sensitivity and high validity and reliability, then the scientific uncertainties inherent in testing and risk assessment could be reduced substantially. In reality, every test is a trade-off between these requirements.

Since every test represents a trade-off between these aspects, researchers face the challenge of combining tests with different strengths and weaknesses, and converting them into scientifically well-founded and resource-efficient test systems, where the tests compensate for each other's weaknesses as much as possible. The integration of the various possibilities offered by *in vivo*, *in vitro* and *in silico* methods is the most mature solution to the knowledge gap, which is huge. Integration can take advantage of the possibilities of each approach.

In regulatory applications, toxicological tests are combined into test systems. A test system contains rules for when and in what order the different tests should be performed. With the resources presently available, it would be necessary to use tiered systems in which relatively simple tests are performed for all chemicals that are up for assessment, and the outcomes of these simple tests are used to prioritise substances for further, more re-

source-intensive testing. Furthermore, efforts towards integration should address a more flexible scheme beyond the classical tiered approach, in which *in silico* screening is the first step, followed by *in vitro* and finally *in vivo*. A feedback mechanism may offer advantages, and multiple parallel inputs may increase understanding.

In future, a large number of data, from *in vivo*, *in vitro* and *in silico* studies, will become available. A strategy to integrate the different methods has to allow for the evolving situation.

Main findings:

The discussion at the workshop addressed the barriers to more efficient integration and the needs to solve current limitations, including language, concepts, formalism and subjectivism.
The discussion identified areas to be evaluated in order to improve the integration between *in vivo*, *in vitro* and *in silico* methods. Some of these efforts should clarify certain aspects of individual methods, allowing a better dialogue between the methods; other efforts are required to better plan the overall integrating scheme.

- Qualify and quantify uncertainties. Each method (*in vivo*, *in vitro* and *in silico*) should produce a result (for instance, a toxicity value or class) with a given defined uncertainty. If such an uncertainty is not defined it will be very difficult to use the result of a given method for the final chemical assessment. Also, the current practice is not satisfactory in this aspect.
- Improve transparency.
- Improve standardisation, achieving a common metric.
- Evaluate advantages and disadvantages of each method: *in vivo*, *in vitro* and *in silico*. It has not been possible, within the workshop, to explicitly list a series of features characterising the different approaches. Some common features apply to the same category of methods, but this is not always the case. Pros and cons of individual methods are related to the target of the integrated system, which may differ.
- Know how results obtained from this test relate to effects on the target system, i.e. humans, and the ecosystems that the regulation intends to protect, so as to determine the predictive value of any test. This, however, is seldom possible. At best, researchers can compare the simple test to a more advanced one, and even this is a far from perfect approximation. Even a state-of-the-art test, such as a long-term animal test, provides in its turn only an estimate of the effects in humans;
- Codify in mathematical terms the common scheme for risk assessment.
- Define the purposes of the integrated system.
- Define a clearer and more formally correct decision analysis, identify criteria and acceptability of decisions.
- Identify the inputs, outputs and scope of the overall scheme. Different strategies are necessary for the different scopes. For instance, if the target of the integration is for regulatory purposes, attention has to be given to false negatives and the thresholds for them. If the purpose of the integration is drug development, false positives have to be minimised.
- Identify a reference for an integrated system. Bodies, such as OECD and ECVAM, address individual methods, but the evaluation of the integrated system requires advanced efforts.
- Identify features for the evaluation of the integrated system, such as cost, friendliness and possibility of automatism.
- Develop integrated *in silico* tools, which will address the overall chemical assessment, targeting ecosystems and improving the current approach in which *in silico* models mimic the existing tests focused on specific species.

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RETHINK

Mini-pigs as models for the toxicity testing of new medicines and chemicals: impact assessment

Contract No	**LSSB-CT-2005-018776**
Project type	**Specific Support Action**
EC contribution	**€ 200 000**
Starting date	**1 March 2006**
Duration	**24 months**
Website	**www.rethink-eu.dk**

Background and objectives:

The objective of RETHINK is to provide an assessment of the mini-pig as an alternative in toxicity testing, and of the potential contribution of the mini-pig to the 3Rs, i.e. replacement, refinement, and reduction in animal testing. Mini-pigs are strains of domestic pigs that are markedly smaller than farmyard varieties, and are thus better adapted to laboratory housing.

The pig closely resembles man in many features of its anatomy, physiology, biochemistry and lifestyle. In particular, the cardiovascular system, skin and digestive tract are considered to be very good models of their human equivalents. Because of these similarities, the toxic effects of chemicals and drugs in pigs may resemble the effects in man more closely than some other commonly used laboratory animals.

If the pig really is a good model for man, then pig and mini-pig cells and tissues could be especially useful for *in vitro* tests and alternative methods. If the mini-pig provides a better predictive model of toxicities to humans than traditionally used animal models, then there could be a significant public health benefit.

Approach and methodology:

The RETHINK partners have assembled groups of experts from around Europe to participate in expert study groups. Guided by the project steering group, these study groups are carefully reviewing the available knowledge base on pigs and mini-pigs. The expert groups are covering different areas and are answering questions about the utility, validity added value and ethics of mini-pigs in toxicity testing.

If the mini-pig is to provide a valid and realistic alternative approach for use in the regulatory testing of new medicines and chemicals, while contributing to the 3Rs, many questions need to be asked. These questions cover 5 different areas:

- welfare and animal care;
- ethical issues;
- development of new medicines and chemicals;
- safety testing;
- genomics and other emerging technologies.

The agenda has been prepared for the expert groups, defining the relevant questions that each group must answer. In addition, they must summarise the state of the art in their area, identify gaps in the knowledge where further research is required, point out technical gaps that may hinder progress, summarise the advantages and disadvantages of the mini-pig, and formulate recommendations regarding the use of mini-pigs in regulatory toxicology and their potential contribution to the 3Rs.

Expected outcome:

The result of the project will be a detailed report, which will provide concrete advice on several points:

- ensuring that the welfare needs of mini-pigs are met in laboratories;
- the scope for the application and development of the 3Rs through the use of mini-pigs;
- the potential role and best deployment of mini-pigs in toxicology testing strategies;
- the validity and added value of mini-pigs in regulatory toxicology;
- proposals for initiatives to fill gaps in knowledge or 'technical gaps';
- implications for the development of new medicines and chemicals.

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SCARLET

Structure-activity relationships leading experts in mutagenicity and carcinogenicity

Contract No	**LSHB-CT-2007-44166**
Project type	**Specific Support Action**
EC contribution	**€ 112 840**
Starting date	**1 June 2007**
Duration	**12 months**

Background and 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 (Quantitative Structure Activity Relationships) foreseen by various legislative initiatives, including REACH, requires that the models fulfil severe quality criteria.

Unfortunately, this ideal situation is not a common occurrence in toxicology. This project is aimed at organising a discussion and a workshop so that leading experts can critically review mutagenicity and carcinogenicity in the field of (Q)SARs, while identifying important issues that require further investigation. The SCARLET project is multidisciplinary and multisectorial, and targets 40 participants; regulators, scientists, problem holders, animal welfare experts, citizen organisations, and the chemical industry will participate. Registration will be free in order to secure a wider participation. Moreover, five fellowships will be awarded to young researchers. The workshop will be useful in overcoming the barriers between science and the general public.

SCARLET will discuss a framework for comparing and integrating individual research activities. Coordinators of EC projects will be invited, and their results will be disseminated, effectively raising the profile of European research. This will also avoid repetition of work, making optimal use of public money and identifying synergisms. The EC projects will be a way to exploit recommendations from the workshop. Verifiable results will include the workshop, the internet site, a list of software and databases, and the final report, which will contain recommendations on the use of computer-based methods in carcinogenicity, mutagenicity and genotoxicity. A paper summarising the workshop discussion will be submitted to a scientific journal.

The main objective is to organise an international scientific workshop with leading experts on QSARs for genotoxicity and carcinogenicity. This workshop will combine the experience of the various scientific communities working in this area; evaluate the current state of the art; identify open research issues; harmonise collaborations within the EC to solve them; and provide scientific guidance for the implementation of genotoxicity and carcinogenicity QSARs for regulatory purposes.

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. More specifically, it will address the requirements of the 7th amendment of the Cosmetics Directive 76/768/EEC and the recent legislative initiative REACH, to characterise the toxicological properties of a large number of

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

The application of QSARs in a regulatory setting is still controversial. Therefore, the SCARLET project partners have identified the following specific objectives for the proposed workshop:

- identification of requirements for QSAR models for genotoxicity and carcinogenicity from the perspective of various stakeholders (scientists, regulators, end users and industries);
- evaluation of existing QSAR models and techniques in respect to these requirements and to the OECD principles for validating QSAR 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 QSARs 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 QSAR models.

In addition, it is likely that the stakeholders will require additional criteria for the acceptance of QSAR models. For this reason, the main scientific and technical objective will be 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 is likely that further scientific or technical topics will have to be addressed, which may include:

- 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 QSAR studies that deal with their complexity and redundancy;
- evaluating the role of QSAR 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 with mechanistic knowledge);
- identifying unresolved research issues within this area and to coordinate research efforts.

These objectives will be addressed within the workshop, in the preparatory discussions of the advisory committee, and by the use of a questionnaire for the stakeholders. The verifiable results will be the workshop, the internet site, a list of suitable software and databases for genotoxicity and carcinogenicity predictions, the final document and the published papers. This outcome also represents the dissemination of the results of this project.

The new EC chemicals legislation REACH 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 QSAR models can play an important role in filling data gaps and providing information for targeted bioassays. Similarly, the 7th amendment of the Cosmetics Directive 76/768/EEC foresees a ban on the testing of cosmetic ingredients on animals, if a validated alternative method is available.

The proposed workshop will contribute to increased information on industrial chemicals and cosmetics, as well as to a reduced number of animal experiments without compromising on public health issues.

Expected outcome:

The expected achievements are:

- an international workshop;
- a document summarising the final results from the workshop;
- a list of suitable databases and software for the issues addressed;
- a series of papers sent to a recognised journal.

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FP7 PROJECTS

ESNATS

Embryonic stem cell-based novel alternative testing strategies

Grant Agreement No	HEALTH-F5-2007-201619
Project type	Integrated Project
EC contribution	€ 11 895 577
Starting date	1 April 2008 (planned)
Duration	60 months

Breakdown and objectives:

ESNATS aims to develop a novel toxicity test platform based on embryonic stem cells (ESC), especially human ESC (hESC), to accelerate drug development, reduce research and development (R&D) costs and propose a powerful alternative to animal tests (3Rs). ESNATS will address the following current drug-testing shortcomings:

- testing takes place late in the development cycle;
- animal test systems bear the risk of non-prediction due to inter-species variation;
- non-ESC assays rely on primary cells or cells of malignant origin that are hard-to-standardise and limited in regard to quantity, homogeneity and genetic diversity;
- existing assay systems based on primary animal cell lines do not reliably represent the physiological situation.

Approach and methodology:

ESNATS will develop a battery of toxicity tests using hESC lines subjected to different standardised culture protocols. Tests will cover embryoid bodies in different developmental stages and differentiated derivatives, including gamete and neuronal lineages, complemented with test systems for hepatic metabolism. Predictive toxicogenomics and proteomics markers will be identified. The individual tests will be integrated into an 'all-in-one' test system. To enable future industrial use, ESNATS will prepare automating and scaling up of hESC culture. The predictivity, quality and reproducibility of ESNATS will be evaluated in a proof of concept study. ESNATS aims towards the following benefits: increase safety due to better predictivity of human test systems; reduce, refine and replace animal tests; lower testing cost; and support medium-, high-throughput testing.

Expected outcome:

ESNATS objectives will be achieved in a five-year multi-disciplinary collaboration of leading European researchers in alternative testing, toxicology, ESC research, genomics, modelling, and automation. The consortium will also include representatives from regulatory bodies, the pharmaceutical industry and ethical advisors to provide guidance to ensure rapid applicability of the developed test systems.

ESNATS

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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 No	**HEALTH-F5-2007-201335**
Project type	**Specific Targeted Research Project**
EC contribution	**€ 3 933 271**
Starting date	**1 April 2008 (planned)**
Duration	**42 months**
Website	**www.nanotest.org**

Breakdown and objectives:

Nanoparticles (NP) have unique, potentially beneficial properties, but their possible impact on human health has not been adequately assessed. The main goal of NANOTEST is to develop alternative high-throughput testing strategies using *in vitro* and in silico methods to assess the toxicological profile of NP used in medical diagnostics. The consortium's specific aims are to: (1) define NP properties and fully characterise NP to be used; (2) study NP interactions with molecules, cells and organs and develop *in vitro* methods to study the toxicological potential of NP; (3) validate *in vitro* findings in short-term in vivo models and study particle effects in animals and (ex vivo) in humans to assess individual susceptibility to NP; and (4) develop in silico models of NP interactions.

Approach and methodology:

Experimental work is structured in four Work Packages (WPs) to address NP characterisation and key elements in the evaluation of NP uptake, exposure and toxicology. NANOTEST integrates the investigation of toxicological properties and effects of NP 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, liver, lung, placenta, digestive and central nervous systems.

As NP action is likely to involve oxidative stress, the consortium will focus on the cross-cutting areas of inflammation, cellular toxicity, immunotoxicity, genotoxicity and related endpoints. Following the development of standard operating procedures and the generation of a common database, in parallel with in silico assays (QSAR, PBPK modeling), NANOTEST will evaluate toxic effects and interactions of NP used in nanomedicine.

Expected outcome:

Results will be validated in an experimental ethically approved in vivo model. The most advanced and standardised techniques will be adapted for automation and prepared for validation by ECVAM. Separate WPs will be dedicated to dissemination and to effective administrative and scientific management.

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OPENTOX

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

Grant Agreement No	**HEALTH-F5-2007-200787**
Project type	**Specific Targeted Research Project**
EC contribution	**€ 2 975 360**
Starting date	**1 April 2008 (planned)**
Duration	**36 months**
Website	**www.opentox.org/projects/opentox/wiki/OpenTox**

Breakdown and objectives:

The goal of the OpenTox project is to develop a predictive toxicology framework with a unified access to toxicological data, QSAR. It will provide tools for the integration of data from various sources (public and confidential), for the generation and validation of QSAR models, libraries for the development and integration of new QSAR algorithms, and validation routines. OpenTox will attract toxicological experts without QSAR expertise, as well as model and algorithm developers. It will move beyond existing attempts to solve individual research issues by providing a flexible and user-friendly framework that integrates existing solutions and new developments.

OpenTox will be relevant for REACH as it gives risk assessors simple access to experimental data, QSAR models and toxicological information that adheres to European and international regulatory requirements.

OpenTox will be published as an open source project to allow a critical evaluation of its algorithms, to promote dissemination, and to attract external developers. Facilities for the inclusion of confidential in-house data and for accessing commercial prediction systems will be included.

OpenTox will contain high-quality data and QSAR models for chronic, genotoxic and carcinogenic effects. These are the endpoints with the greatest potential to reduce animal testing. The impact of OpenTox will, however, go beyond REACH and long-term effects because create models for other endpoints (e.g. sensitisation, liver-toxicity, cardio-toxicity, ecotoxicity).

Expected outcome:

The proposed framework will support the development of new QSAR models and algorithms by automating routine tasks, providing a testing and validation environment, and allowing the easy addition of new data. For this reason, the project partners expect that OpenTox will lead to QSAR models for further toxic endpoints and generally improve the acceptance and reliability of (Q)SAR models.

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PREDICT-IV

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

Grant Agreement No	**HEALTH-F5-2007-202222**
Project type	**Integrated Project**
EC contribution	**€ 11 330 907**
Starting date	**1 April 2008 (planned)**
Duration	**60 months**

Breakdown and objectives:

The overall aim of PREDICT-IV is to develop strategies to improve the assessment of drug safety in the early stage of development and late discovery phase by an intelligent combination of non animal-based test systems, cell biology, mechanistic toxicology and in-silico modelling – in a rapid and cost effective manner. A better prediction of the safety of an investigational compound in early development will be delivered. Margins-of-safety will be deduced and the data generated by the proposed approach may also identify early biomarkers of human toxicity for pharmaceuticals. The results obtained in PREDICT-IV will enable pharmaceutical companies to create a tailored testing strategy for early drug safety.

The project will integrate new developments to improve and optimise cell culture models for toxicity testing and to characterise the dynamics and kinetics of cellular responses to toxic effects *in vitro*. The target organs most frequently affected by drug toxicity will be taken into account, namely liver and kidney. Moreover, predictive models for neurotoxicity are scarce and will be developed. For each target organ the most appropriate cell model will be used. The approach will be evaluated using a panel of drugs with well-described toxicities and kinetics in animals and partly also in humans.

This approach will be highly advantageous, as it will allow a direct comparison between the in vivo and *in vitro* data. A parallel analysis of several dynamic and kinetic models with a broad spectrum of endpoints should allow for the identification of several relevant biomarkers of toxicity. Inter-individual susceptibilities will be taken into account by integrating the polymorphisms of the major drug metabolising enzymes and correlating the observed effects in the human cell models with their genotype. Environmental influences on cellular toxicity to these compounds will also be evaluated using hypoxic stress as a relevant test model.

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START-UP

Scientific and technological issues in 3Rs alternatives research in the process of drug development and Union politics

Grant Agreement No	**HEALTH-F5-2007-201187**
Project type	**Specific Support Action**
EC contribution	**€ 317 964**
Starting date	**1 March 2008**
Duration	**24 months**

Breakdown and 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 in the 3Rs in Europe, 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 candidates for further development and avoiding efforts for under-performing candidates are essential for the competitiveness of 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 biological entities.

Approach and methodology:

The project is structured around three workshops, preceded by two expert meetings redefining and prioritising current bottlenecks in the 3Rs methodology, drug discovery and development. The limitations and gaps of each phase will be addressed, e.g. many cell systems do not have the required stability for genomics, proteomics or metabonomics and many current cell systems 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 will be proposed.

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Alternative Testing Strategies

Replace the need for animal experiments, reduce the number of animals required and refine techniques are the three concepts underlying the development of alternative testing strategies used in pharmaceutical discovery and development, and in safety assessment of chemicals. Research into alternative testing strategies formed part of the EU's Sixth Framework Programme (2002-2006) and the purpose of this catalogue is to demonstrate the activities initiated over the duration of the programme and the initial results generated. A particular focus of the work is the development of *in vitro* methods and on methods which can be validated to international standards, achieve approval of regulatory authorities and be adopted by industry.

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