



The European Partnership  
for Alternative Approaches to Animal Testing

# ABSTRACTS BOOK

## EPAA 2008 CONFERENCE

***Research into Alternative Approaches: Are we on the right track?***

Brussels, 3 November 2008



## QSAR models for bioconcentration and fish toxicity for regulatory purposes

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Abstract	<p>Quantitative-structure active relationship (QSAR) models can be used to replace animal models. Their use so far has been questioned for the possible occurrence of errors, in particular of the so-called false negatives: predictions as safe, while the chemical it is not.</p> <p>In case of models for regulatory purposes, particular care has to be given to the quality of the input data (check of structures and values), to the validation of the model, and to the use of the model results. Typically QSAR models have been evaluated using squared errors, thus the information on the false negatives or positives is lost.</p> <p>We developed models with particular attention to these key factors. We show here two examples. In the case of QSAR models for aquatic toxicity typically the results are wrong for the most toxic compounds, which is the worst situation for regulators. In the past we developed a QSAR model for pesticide toxicity, DEMETRA, and now we used it to predict toxicity of industrial chemicals, assessed according to the Verhaar scheme, as implemented in the software ToxTree developed by ECB. DEMETRA gave much better results than other software, such as ECOSAR, TOPKAT, Dragon, considering the false negatives.</p> <p>Furthermore, we developed another QSAR model within the project CAESAR for bioconcentration in fish (BCF). BCF is very important for REACH and for classification and labelling.</p> <p>The predictions from CAESAR have been shown to be superior to those of other software, such as ECOSAR. We further checked the results of CAESAR with the EURAS database. Good predictions have been confirmed.</p> <p>Thus, QSAR can replace animal models for REACH.</p> <p><b>ACKNOWLEDGEMENTS</b></p> <p>We acknowledge the EC project CAESAR.</p>

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**In view of Replacement**

## Non-animals tests for estrogenic, androgenic, thyroid, progesteric and glucocorticoid-like compounds by CALUX bioassays

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Abstract	<p><b>RESULTS</b></p> <p>Current used animal tests for infertility and reproductive toxicology are the Hershberger and Allen Doisy test. In the FP6 EC Project ReProTect these methods have been compared to non-animal alternatives such as in vitro reporter gene assays ER and AR CALUX. It is virtually impossible to analyse all chemicals in a cost and time effective manner with animal testing – therefore a paradigm shift to more bioeffect-based screening technology is required. Current emphasis of endocrine disruptive chemicals (EDC) research is mostly on the measurement of estrogenic and androgenic compounds. In this study, a panel of CALUX<sup>®</sup> bioassays testing for estrogen (ER<math>\alpha</math> CALUX<sup>®</sup>), androgen (AR CALUX<sup>®</sup>), thyroid (AR CALUX<sup>®</sup>), progesterone (PR CALUX<sup>®</sup>), and glucocorticoid (GR CALUX<sup>®</sup>) are described and applied on various chemical and pharmaceutical compounds. The comparison between rat in vivo tests called Allen Doisy /Hershberger for estrogens/androgens versus the in vitro human ER<math>\alpha</math>- and AR CALUX for various pharmaceuticals and chemicals showed a significant correlations between animal and non-animal test with ratios of R<sup>2</sup> values of 0.87 (Allen Doisy vs. ER<math>\alpha</math>-CALUX) and 0.46 (Hershberger vs. AR-CALUX). Finally standard operation protocols for all the assays have been established in the ReProTect project. Tests on robustness and stability of the ER<math>\alpha</math> CALUX and AR CALUX are executed by several laboratories. A new set of chemicals for prevalidation has been put forward by ECVAM. Currently, the inter-laboratory transferability of the CALUX tests is evaluated within different laboratories. These results show that the CALUX<sup>®</sup> panel can successfully be applied to such compounds as well as to complex mixtures of these compounds and that different types of hormone receptor agonistic and antagonistic activity could be detected.</p> <p><b>SUMMARY</b></p> <ul style="list-style-type: none"><li>▪ Our project relates mostly to the reduction and even replacement of the animal tests for reproductive toxicology</li><li>▪ After 2013 reproductive testing is mandatory for chemicals and pharmaceuticals with more than an annual production of 1000 t. Therefore our aim is to offer a cost-effective and fast alternative for reproductive toxicity testing (cost reduction &gt; 80%; TAT 15 d).</li><li>▪ This bioassay panel approach has been applied in a wide range of European Framework projects such as FIRE, ReProTect, NewGeneris, Techneau and ACE.</li></ul> <p><b>ACKNOWLEDGEMENTS</b></p> <p>The authors would like to acknowledge the EU for funding the ReProTect and other projects wherein the panel of CALUX tests have been evaluated.</p>
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Reduction - In view of Replacement

[Link to the poster](#)

## QSAR modelling of biological activity by descriptors calculated with simplified molecular input line entry system (SMILES)

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Abstract	<p>Quantitative structure – activity relationships (QSAR) models are tools for the prediction of biological endpoints without animal testing. QSAR methods are highly dependent on the input data, not only on their quality but also on the form they are fed to the model. Typically the chemical information is given to the QSAR in the form of molecular descriptors. Here we present QSAR models based on the search of strings invariants within the simplified molecular input line entry system (SMILES) code. This information is widely available on the internet for databases of chemical compounds. We have studied the ability of optimal SMILES-based descriptors for QSAR modelling of carcinogenicity, mutagenic potentials, and toxicity towards E. Coli bacteria for nanosized oxides (experimental data was obtained in our laboratory). The model we obtained gave good prediction, modelling carcinogenicity and mutagenicity of diverse chemicals, and toxicity against E. Coli of nanomaterials. The predictions are quantitative, while in many cases the models for carcinogenicity and mutagenicity are only for categories. This shows good possibilities of a new modelling approach which can be used for screening of chemicals and nanomaterials, replacing animal models.</p> <p><b>ACKNOWLEDGEMENTS</b> We acknowledge the EC projects CHEMPREDICT and CAESAR.</p>

[Link to the poster](#)

In view of Replacement

# In vitro Fertility Assessment by Multi-parametric Tissue Culture Bioassays

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Abstract	<p>There is a need for predictive in vitro screening methods for compounds affecting the reproductive function. Procreation is an integrated process involving the fertility of two individuals (male and female) of which in each the normal functionality depends on the time dependent interplay between intricate processes of the different organs systems. Simple cell culture systems can indeed not provide a lot of useful information to predict the impact of a compound on the reproductive health of an individual. To screen compounds for their impact on fertility and reproductive health the complete life cycle needs to be evaluated. The classical reproductive toxicity studies (OECD guidelines) look for adverse effects on sexual function and fertility in male and female and developmental toxicity on the offspring. These long term in vivo experiments use thousand of animals and deduce effects from secondary end parameters such as libido, sexual behaviour, implantation, number of offspring, normality and health of the life young and their capacity to procreate. However tissue cultures that represent the function of the target organ with multi-parametric primary end points can provide a good in vitro alternative. We focussed on the development on innovative tissue culture methods for in vitro reproductive health assessment. Our established follicle bioassay (FBA) mimics the ovarian function and provides for the simultaneous evaluation of folliculogenesis, steroidogenesis and oogenesis. Coupled with the in vitro maturation assay (IVM), evaluating the meiotic process of the oocyte and an in vitro fertilization procedure (IVF) to evaluate perturbation of the conception process, important aspects of female fertility can be screened. In addition, the effect on pre- and peri-implantation embryo development can be assessed by our Mouse Embryo Peri-implantation Assay (MEPA), which daily evaluates the survival and development capacity of the embryo. By applying these assays in a test battery, the 4R principle for laboratory animal use; Replacement, Reduction, Refinement and Relevance can be implemented for the screening of compounds for female fertility. In vitro bioassays focussing on male reproductive organs are under development.</p> <p><b>ACKNOWLEDGEMENTS</b> EU FP6 ReProTect project (pre-validation of the FBA).</p> <p><u>References of examples:</u> - <a href="#">Van Wemmel K</a>, <a href="#">Gobbers E</a>, <a href="#">Eichenlaub-Ritter U</a>, <a href="#">Smits J</a>, <a href="#">Cortvrindt R</a>. Ovarian follicle bioassay reveals adverse effects of diazepam exposure upon follicle development and oocyte quality. <i>Reprod Toxicol</i>. 2005 Jul-Aug;20(2):183-93. - Van Merris V., Van Wemmel K., Cortvrindt R. In vitro effects of dexamethasone on mouse ovarian function and pre-implantation embryo development. <i>Reproductive toxicology</i> 2007; 23(1):32-41 - <a href="#">Lemeire K</a>, <a href="#">Van Merris V</a>, <a href="#">Cortvrindt R</a>.- The antibiotic streptomycin assessed in a battery of in vitro tests for reproductive toxicology. <i>Toxicol In Vitro</i>. 2007 Oct;21(7):1348-53.</p>

[Link to the poster](#)

Replacement

## Hepatocyte-like cells from human skin: a promising tool for *in vitro* pharmaco-toxicological research and testing

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Abstract	<p>The withdrawing of promising drug candidates is often triggered by the detection of hepatotoxicity in (pre)clinical studies. The availability of reliable <i>in vitro</i> screening models capable of detecting hepatotoxicity, and in particular chronic liver toxicity, in an early stage of the drug development process is thus of utmost importance for the industry. Today, however, most existing liver-based models suffer from phenotypic instability and are rodent-derived. Hence, they are not fully representative for the human situation <i>in vivo</i>. The development of a model based on easily obtainable human adult stem cells could overcome this problem and as such reduce the number of laboratory animals involved. Indeed, functional human liver cells (hepatocytes) can be produced out of adult stem cells from tissues of different origin. This way of working could, once industrially optimized and produced in routine, replace the currently -widely used- animal-based models. In previous research, we have already shown that stem cells isolated from adult human bone marrow are able to successfully differentiate into functional liver-like cells upon sequential and gradual exposure to hepatogenic factors, reflecting the pattern of liver development <i>in vivo</i>. Since human bone marrow, however, remains a difficult accessible source of adult stem cells, we investigate here whether human adult stem derived from easier accessible sources, such as human skin, are able to differentiate into similar functional liver-like cells. Preliminary results show that freshly isolated stem cells from human foreskin express important stemness markers and both typical biliary and liver markers. Upon sequential and gradual exposure to hepatogenic factors, cells undergo morphological changes, characterized by a fibroblastic to epithelial transition and enlargement of the nuclei. Differentiation also occurs at the protein level. Indeed, important liver-specific markers are expressed from about 20 days onwards. Later on, the expression of foetal and adult liver markers decreases and increases, respectively, thereby closely reflecting the patterns observed during <i>in vivo</i> liver development. In conclusion, our preliminary skin results show that we can isolate cells that exhibit stem cell-like features and that are capable to differentiate into liver-like cells upon mimicking the <i>in vivo</i> micro-environment of the developing liver cell in culture. Once fully developed, this methodology could represent a promising tool for <i>in vitro</i> pharmaco-toxicological research and testing of new compounds and replace and/or reduce existing animal models.</p> <p><b>ACKNOWLEDGEMENTS</b></p> <p>This work was supported by grants from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen), Belgium; the Fund for Scientific Research (FWO) Vlaanderen, Belgium; BruSTEM, Brussels, Belgium; The 6<sup>th</sup> European Framework Liintop (project n° 037499); and the European 7<sup>th</sup> Framework program ESNATS (project n° 201619).</p>

[Link to the poster](#)

**In view of Replacement – Reduction**

## Further evaluation of the HET-CAMVT assay to assess vascular toxicity

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Abstract	<p><b>INTRODUCTION</b></p> <p>A significant hazard of intravenous (i.v.) drug administration is vascular irritation, a side effect that can impose relevant delays in preclinical and clinical drug development. The standard HET-CAM (Hen's Egg Test - Chorioallantoic Membrane), an assay routinely used to identify the eye irritating potential of compounds (accepted by the EU National Regulatory Authorities), was modified in an attempt to predict injection site irritation observed during in vivo i.v. studies. After treatment, a detailed evaluation (14 subclasses) of the standard endpoints (haemorrhage, lysis and coagulation) within the first 5 minutes and then after 15, 30, 45 and 60 minutes was performed by using a stereomicroscope. The observed effects were quantified by calculation of a Vascular Irritation Index based on an 'in house developed' scoring-system.</p> <p><b>RESULTS</b></p> <p>The in vitro observed irritation effects were reproducible and concentration- and time-dependent, which allowed us to rank the tested formulations. For each of the projects, the predictive capacity of the HET-CAM<sup>VT</sup> (HET-CAM-Vascular Toxicity) was shown by correlating these in vitro data with effects observed at the injection site during i.v. dosing in preclinical in vivo studies. Based on these results we can conclude that the HET-CAM<sup>VT</sup> approach allows excluding moderately to severely i.v. irritating formulations from animal experiments and lead to a reduction of animal numbers required for local tolerance testing.</p>
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[Link to the poster](#)

Reduction

# Aiming at international acceptance of evident toxicity as an endpoint in acute inhalation toxicity tests

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Abstract

## BACKGROUND

People may be exposed to chemicals when they breathe (by inhalation) either deliberately when using an inhaled medicine, inadvertently when using chemicals at work or in the home, or indirectly as a result of contamination of the atmosphere. At present, the only way to test for potential hazards from such exposures and establish safe limits is to observe the effects on living animals, usually rats. The OECD Test Guidelines are a collection of internationally agreed methods used to assess the hazards of chemicals. Under the OECD Mutual Acceptance of Data Agreement of 1981, Regulatory Authorities in Member Countries have agreed to accept data obtained in accordance with OECD Test Guidelines providing the OECD principles of Good Laboratory Practice were observed. As such, the Test Guidelines are a powerful tool for setting standards for testing and reducing duplication world-wide.

There is an existing 1981 acute inhalation toxicity Test Guideline (TG 403). This, like the now deleted 1981 acute oral guideline TG 401, could be modified to a protocol that uses fewer animals (Reduction) and does not involve such severe effects (Refinement). Therefore an initiative was taken to develop a revised Test Guideline.

## THE PROPOSED TEST GUIDELINE

The UK proposed a revised Test Guideline known as the Fixed Concentration Procedure (FCP, draft TG 433). Using the FCP, a decision on whether further testing is required at higher, and potentially more toxic concentrations, is made by assessing the animals for signs of toxicity, termed “evident toxicity”, rather than relying on severe toxicity or deaths. An example of the protocol will be presented in the poster.

## REGULATORY ACCEPTANCE: NON-LETHAL ENDPOINTS AT OECD

One of the key stumbling blocks to the OECD adoption of TG 433 is the reluctance to accept evident toxicity as a reliable endpoint. This is despite the OECD acceptance of evident toxicity as a validated endpoint for oral toxicity testing. A small consortium was therefore established to develop the information and arguments required to support the international adoption of non-lethal toxicity as a valid endpoint in acute inhalation toxicity testing. Thus the project is related to Refinement.

## RESULTS

Identification of the key steps:-

- Identify the root causes of the concerns expressed
- Develop arguments/ evidence to meet these concerns

Once the above have been completed:-

- Discuss the evidence-based case with European partners to obtain support and use of the method within REACH
- Re-launch of TG 433 as an OECD Test Guideline proposal

Several causes of concern have been identified and work is in progress to address these as follows:-

- legal/regulatory requirements within the EU have been summarised
- conceptual difficulties and inertia to be met by scientific reasoning and experience with the oral test guidelines
- clarification of the term “evident toxicity” will be the subject of a new project initiated with NC3Rs funding
- historical data will be used for comparison of the modeled outcomes of the proposed TG 433 with those of the existing TG 403, also with NC3Rs funding.

## REFERENCES

OECD (2000). Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluation. Environmental Health and Safety Monograph Series on Testing and Assessment No. 19. Available: :

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OECD TG 420

Van den Heuvel, M.J., Clark, D.G., Fielder, R.J., Koundakjian, P.P., Oliver, G.J.A., Pelling, D., Tomlinson, N.J. and Walker, A.P. (1990). The international validation of a fixed-dose procedure as an alternative to the classical LD<sub>50</sub> test. *Fd. Chem. Toxicol.* **28**, 469-482.

[Link to the poster](#)

**Reduction and Refinement**

## Value of the mouse carcinogenicity study and the 1-year dog study to pesticide hazard and risk assessments

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Abstract	<p>Previous reviews of the value of the mouse cancer bioassay, most recently the ILSI-HESI ACSA Task Force, have concluded that the study does not add significant additional information for carcinogenicity evaluation over and above the use of the carcinogenicity study in the rat. In this assessment of 202 pesticides evaluated in the EU review under Directive 91/414/EEC, the conclusions reached by ACSA were confirmed in that the data provided by the mouse carcinogenicity studies contributed little or nothing to ADI-setting, human health risk assessments or classification and labeling (with just a total of 1.5% being classified as carcinogens, all in Category 3). From the perspective of EU regulatory decision making on pesticides, the mouse data did not influence a single decision. Approximately 100,000 mice were used in studies on these pesticides with negligible benefit in either risk or hazard assessment for humans. This review reinforces the need from a scientific and animal welfare perspective to drop the mouse carcinogenicity from Directive 91/414/EEC data requirements.</p> <p>A review of pertinent publications on pesticides dealing with the need of running dog studies and their duration was performed. A substantial number of publications confirm the need for systemic toxicity tests in a second species (non-rodent) in the safety dossier for agrochemicals. Three key publications with different approaches investigate the value of the 12-month dog study in addition to a 3-month study. The need for a repeat dose study is not debated. However, and despite different data bases and approaches all conclude with the recommendation to limit dog testing with pesticides to 3-month studies. A synthesis of these reviews is performed and the conclusion is drawn that a 12-month study in addition to a 3-month study adds very little to the safety assessment of pesticides. It is recommended to abandon the requirement for a 12-month dog study and adapt the pesticide legislation where it is still a requirement.</p>

### Reduction and Refinement

[Link to the poster](#)

## RETHINK project: the impact of the minipig in toxicity testing

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Abstract	<p><b>OBJECTIVE</b></p> <p>In the main regulatory frameworks for pharmaceuticals and food testing in a non-rodent species is required, and the default choice is mainly dog and nonhuman primate. The objective of the RETHINK project is to provide an assessment of the minipig as an alternative for dog and especially nonhuman primate in toxicity testing, and in this respect the potential contribution of the minipig to the 3R's (replacement, refinement, and reduction in animal testing). The pig closely resembles man in many features of its anatomy, physiology, biochemistry and lifestyle. Because of these similarities the toxic effects of chemicals and pharmaceutical drugs in pigs may resemble the effects in humans more closely than do some other commonly used laboratory animals, such as the dog or rabbit. To provide a valid and realistic approach for use of the minipig in the regulatory testing of new medicines and chemicals, while contributing to the 3Rs, many questions are raised. The project has assembled groups of experts from around Europe to participate in working study groups being in the process of a careful review of available knowledge base on pigs and minipigs and address questions about the utility, validity added value and ethics of minipigs in toxicity testing. The project will result in a detailed report with concrete indications on issues such as welfare needs of minipigs in laboratories, scope for application and development of 3Rs, the potential role and best deployment in safety testing strategies, the validity in regulatory toxicology, proposals for initiatives to fill gaps in knowledge or "technical gaps" and implications for development of new medicines and chemicals.</p> <p><b>RESULTS</b></p> <p>The contribution of minipigs to the 3R's is rather Refinement and Reduction than Replacement.</p> <p>Minipigs cannot be considered as "more acceptable" than dogs or monkeys either on grounds of lesser sentience or on the basis of a popular belief that food animals somehow matter less than pets or primates. Therefore "Replacement" is not applicable, but the breeding infrastructure of minipigs may allow some "Reduction". Careful genetic management and opportunities for improvement of the minipig may lead to a better predictive model for humans, provide options for more pertinent testing (e.g. on skin toxicity testing) and less wasteful use of animal resources; and these improvements can be seen as "Refinement" for the preclinical safety testing. The early selection of the most-human-like species for toxicity studies avoids duplications of the non-rodent species and allows a more relevant preclinical strategy, which can be seen as "Reduction".</p> <p>In general there are no restrictions to use minipigs for regulatory toxicity testing. For some non-pharmaceutical product categories the dog is expressed to be the preferred species, but this is mainly based on historical experience rather than on a scientific rationale.</p> <p><b>ACKNOWLEDGEMENTS</b></p> <p>RETHINK is a multinational project, co-funded by the European Commission FP6 Framework Programme under contract number: LSSB-CT-2005-018776.</p>

[Link to the poster](#)

Reduction and Refinement

## A cell-based *in vitro* alternative to identify skin sensitizers by gene expression

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Abstract	<p>Skin allergy in humans (allergic contact dermatitis) is an important occupational and consumer health problem, therefore the identification of substances that may cause a toxicological hazard is of high significance. Currently, the OECD guidelines for skin sensitization are all animal-based, despite the ethical and economic burden associated with animal testing no validated <i>in vitro</i> or <i>in silico</i> method exists. In the context of an expected increase of testing demand due to the European regulation of REACH, the 7th Amendment to EU Cosmetics Directive which includes a marketing ban for finished products and ingredients if animals tests are used and in the context of European legislation on restrictions for animal testing, the need for the development and validation of an alternative test is increasingly compelling.</p> <p>In a previous study at our institute it was demonstrated that human dendritic cells derived from cord blood express specific gene profiles upon exposure to low molecular weight sensitizing chemicals. Building on these findings and after an extended set of gene expression measurements, the VITOLENS assay was constructed [1]. It is a classification model that discriminates sensitizing chemicals from non-sensitizing chemicals based on transcriptome analysis after exposure of dendritic cells. The assay was tested on a set of 21 chemicals (10 sensitizers, 11 non-sensitizers) of which the sensitizing character was known from other (<i>in vivo</i>) sources. The assay predictions were in close concordance with the a-priori knowledge and resulted in a specificity and sensitivity around 90%. These results show the potential of VITOLENS to contribute to the replacement of existing <i>in vivo</i> regulatory toxicological tests for skin sensitization. Further steps to validation of the assay are initiated, the extended protocol has been communicated with ECVAM and the planning of prevalidation is ongoing (transferability, between-laboratory variability, predictive capacity).</p> <p>[1] Jef Hooyberghs, Elke Schoeters, Nathalie Lambrechts, Inge Nelissen, Hilda Witters, Greet Schoeters, Rosette Van Den Heuvel. A cell-based <i>in vitro</i> alternative to identify skin sensitizers by gene expression. Toxicology and Applied Pharmacology 231 (2008) 103–111.</p>

[Link to the poster](#)

Replacement

## Defining conceptual paradigms for an optimization of chemicals testing under REACH: A decision analytic ITS

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Abstract	<p>REACH will expose information gaps and require subsequent potential large safety testing program. REACH reflects a strong policy preference for a more comprehensive, transparent and efficient data acquisition on the risks of chemicals, which is considered a prerequisite for an improved risk management system. This management system, given resources scarcity and encouragement to use animal alternatives, needs to evolve from a checklist approach to transparent and structured frameworks for data integration. The framework, an operational expression of Integrated Testing Strategy, should incorporate data from variety of testing methods. As each test carries different error the framework should be designed to reduce uncertainty with updated information. In return the impact of any missing or inferred information on the reliability of the decision-making process should be quantified. While the concrete structure of ITS may vary considerably across endpoints and even within endpoints, there are common compositional characteristics which clearly distinguish an ITS from tiered testing strategies. We suggest a set of key conceptual requirements for ITS in order to come to a common understanding of ITS and to strengthen their function as efficient hazard and risk assessment tools of chemicals. We propose decision analytic Bayesian approach to ITS and discuss how it meets the desired characteristics. The benefit of adopting a Bayesian approach is that it gives a formal mechanism for combining prior knowledge with the new evidence presented, quantifies dependence between tests and overall uncertainty for inference.</p> <p><b>ACKNOWLEDGEMENTS</b></p> <p>This work was supported by the EU 6th Framework Integrated Project OSIRIS (contract no. GOCE-ET-2007-037017). <a href="http://www.osiris-reach.eu">http://www.osiris-reach.eu</a></p>

[Link to the poster](#)

**Replacement, Reduction, Refinement**

## Tools to understand the cross-talk between keratinocytes and dendritic cells after challenge with contact sensitizers

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Institutions/Companies	L'Oréal Recherche, Aulnay-sous-Bois, France
Abstract	<p>Allergic disease resulting from industrial, environmental or domestic exposure to sensitizers is the most common manifestation of immunotoxicity in humans. Allergen risk assessment and more precisely skin sensitizing potential of ingredients used in the cosmetic and pharmaceutical industries so far essentially depends on <i>in vivo</i> animal models. In the context of the 7th amendment to the Cosmetic Directive as well as the recent EU-legislation on chemicals (REACH), the cosmetic industry is particularly concerned by the challenge of finding <i>in vitro</i> alternatives to assess the sensitizing potential of chemicals. This is also the main objective set up by the European project Sens-it-iv (LSHB-CT-2006-018681) in which we are involved.</p> <p>When developing cell-based test systems at least two major points have to be considered: (1) to acquire a good understanding of the <i>in vivo</i> players and the <i>in vivo</i> mechanisms, (2) to replace as far as possible the <i>in vivo</i> players by cells that are easy to obtain and to standardize as a prerequisite to ensure reproducibility of <i>in vitro</i> tests. Therefore we focused our studies on the understanding of the cross-talk between keratinocytes (KC) and dendritic cells (DC) of the epidermis, i.e. the Langerhans cells (LC), when exposed to contact sensitizers by using the DC surrogate cell line MUTZ-3. With the aim to identify a protocol reproducing at most <i>in vivo</i>-like KC/DC interactions, we first set up co-culture conditions between epidermal equivalent (EPISKIN) and MUTZ-3 cells in order to compare the phenotypical and biochemical changes in the MUTZ-3 cells after direct or indirect exposure to contact sensitizers.</p>

**In view of Replacement**

[Link to the poster](#)

## Towards the use of biochip technologies for the prediction of in vivo acute toxic potential

Authors	<b><i>Note R, Eilstein J, Thomas M, Duché D, Ouedraogo-Arras G, Meunier JR.</i></b>
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Institutions/Companies	L'Oréal Recherche, 1 Avenue Eugène Schueller, 93600 Aulnay-sous-Bois, France
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Abstract	<p>Developing alternative methods to animal testing is critical to the cosmetic industry based on ethical reasons, the REACh regulation and the 7th Amendment of the European Cosmetic Directive. This directive foresees a marketing ban of cosmetic products containing ingredients or combinations of ingredients which have been tested, among other toxicity endpoints, in acute toxicity studies after March 2009.</p> <p>With regards to acute toxicity, considerable efforts have been gathered over the last years to develop in vitro methods that would be sufficiently robust to reduce or even replace in vivo animal testing. The rationale behind derives from observations, notably arising from the Multi-centre Evaluation of In Vitro Cytotoxicity (MEIC) study, that basal cytotoxicity can be used as a surrogate endpoint for predicting lethal systemic toxicity in vivo. Because of their strong commitment to alternative to animal testing, l'Oréal and Solidus Biosciences Inc. have completed a proof of concept study of the Solidus biochips as an alternative to acute toxicity tests. Solidus researchers have developed a high-throughput, automatable technology which can help revealing toxicity potentially mediated by metabolic activation without the use of animals. Such a technology, integrating a cell-based (Datachip) and enzymatic systems (Metachip), provides mechanistically-based information; which is in agreement with the paradigm shift quoted in the document released last June by the United States National Academies of Sciences: "Toxicity testing in the 21<sup>st</sup> century: a vision and a strategy" and in the ToxCast program.</p> <p>In the current study, 50 chemicals representative of L'Oreal's chemical space and cosmetic application have been collated and tested. These compounds ranged in their degree of in vivo toxicity, as defined by the LD50 values and also in their mechanisms of toxic action. Other selection criteria relate to the target species and the route of administration: data generated in rats treated by gavage have been used to assess the correlation between the experimentally derived IC50 and LD50 values. Preliminary statistical analysis has led to encouraging results, with sensitivity and specificity values over 85%. This is an exploratory work and further investigations such as assessing the relevance of such an approach in an Integrated Testing Strategy (ITS) workflow, are still in progress.</p>
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## In vitro micronucleus assay using a co-culture system: towards new tools for in vitro risk assessment of dermally applied compounds?

Authors	<b><i>Gladys Ouédraogo, Michèle Feltès, Linda Bourouf, Nicole Flamand, Jean-Roch Meunier</i></b>
Institutions/Companies	L'Oréal (Aulnay sous Bois) (FR)
Abstract	<p><i>In vitro</i> reconstructed skin models such as Episkin<sup>®</sup> (reconstructed epidermis) and Realskin (reconstructed full thickness: epidermis + living dermis) are biological models mimicking human skin. They are of growing interest in safety or efficacy pre-screening tests and for regulatory purposes as alternatives to animal testing (7th amendment to the European Cosmetic directive, REACH). The reduction and eventually the replacement of <i>in vivo</i> toxicity testing require the development of new complementary biological models and methods with improved ability to predict the genotoxic or other endpoint risk with <i>in vitro</i> data. This can be achieved if these new assays take into account the exposure conditions in a more relevant way than the current ones. To that end, new approaches using human reconstructed skin models for <i>in vitro</i> toxicology assessment are proposed.</p> <p>The skin is the target organ for dermally exposed compounds or environmental stressors. A co-culture system using Episkin<sup>®</sup> or RealSkin and target cells to perform a regular micronucleus assay is used with six different compounds. This way of using human reconstructed skin for genotoxicity testing aims at improving the relevance of exposure conditions in <i>in vitro</i> genotoxicity assays for dermally applied compounds. The skin is indeed a biologically active barrier driving the exposure to compounds and their possible metabolites. The exposure of the target cells to a given substance can be assessed after topical application as was the case here. Episkin<sup>®</sup> and Realskin were used as a metabolically active tissue and a physiologic barrier. The test compound can be metabolized by the skin and/or by the target cells (<math>\pm</math> S9 if needed). Metabolism is an important event to consider in genotoxicity and skin sensitization evaluation. Compared to cell models, a broad variety of chemicals with different physico-chemical features can be evaluated in this system (after topical or systemic application): compounds with different pH, physical state (liquids, gels, solids, formulations).</p> <p><b>REFERENCES</b></p> <p>Flamand, N., Marrot, L., Belaidi, J. P. et al. (2006). Development of genotoxicity test procedures with Episkin<sup>®</sup>, a reconstructed human skin model: Towards new tools for <i>in vitro</i> risk assessment of dermally applied compounds? <i>Mutat. Res.</i> 606(1-2), 39-51.</p> <p>George, E., Westmoreland, C. (1991). Evaluation of the <i>in vivo</i> genotoxicity of the structural analogues 2,6-diaminotoluene and 2,4-diaminotoluene using the rat micronucleus test and rat liver UDS assay. <i>Carcinogenesis</i> 12(12), 2233-2237.</p> <p>Flamand, N., Marrot, L., Belaidi, J-Ph. et al. (2006). <i>Mutation Research</i> 606, 39-51.</p> <p>ICH, Topic S2B (1997). <i>Genotoxicity: A standard battery for genotoxicity testing of pharmaceuticals</i>. International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. Step 4 guideline.</p> <p>TGD (2003). <i>Technical guidance document, edition 2</i>. Joint Research centre, Italy: European Chemicals bureau.</p>

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[Link to the poster](#)

# The COLIPA Strategy for Developing and Pre-validating In Vitro Alternatives for Skin Sensitization Testing

Authors	<b><i>Marrec-Fairley M.<sup>1</sup>, Aeby P.<sup>2</sup>, Ashikaga T.<sup>3</sup>, Bessou-Touya S.<sup>4</sup>, Diembeck W.<sup>5</sup>, Eschrich D.<sup>6</sup>, Gerberick F.<sup>7</sup>, Maxwell G.<sup>8</sup>, Ovigne J-M.<sup>9</sup>, Sakaguchi H.<sup>10</sup>, Tailhardat M.<sup>11</sup>, and Teissier S.<sup>9</sup></i></b>
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Abstract	<h2>RESULTS</h2> <p>Allergic contact dermatitis is a delayed-type hypersensitivity reaction induced by small reactive chemicals (haptens). Currently, the sensitizing potential of chemicals is usually identified on the basis of animal studies, such as the local lymph node assay (LLNA) or Guinea Pig tests. There are, however, increasing public and political concerns regarding the use of animal testing for the screening of new chemicals. Consequently, the development of <i>in vitro</i> models for predicting the sensitizing potential of new chemicals is receiving widespread interest.</p> <p>The COLIPA Skin Tolerance project team aim is to achieve full <b>replacement</b> for the skin sensitization endpoint through the funding of fundamental/applied research projects and the coordination of method development/evaluation activities. COLIPA currently collaborates with a number of academic research groups to increase and apply our understanding of the molecular and cellular events occurring during the acquisition of skin sensitization. At present fundamental and applied research is being funded in the following key areas; chemistry/peptide binding, skin metabolism, skin bioavailability of skin sensitizing chemicals; evaluation of different biomarkers for Dendritic cell activation by chemical sensitizers; and T cell proliferation. Knowledge gained from this research is being used to support the development and pre-validation of novel <i>in vitro</i> approaches for the identification and characterization of skin sensitizing chemicals. At present three <i>in vitro</i> test methods (Direct peptide reactivity assay, MUSST and hCLAT) are being evaluated for their potential to predict skin sensitization potential within COLIPA interlaboratory ring trials.</p> <h2>ACKNOWLEDGEMENTS</h2> <p>COLIPA currently funds skin sensitization research at the following institutions: Hôpital E. Herriot, Lyon, France; Imperial College London, UK; University of Cincinnati, USA; University of Dusseldorf, Germany; Université Louis Pasteur, France. The COLIPA Skin Tolerance project team has ongoing collaborations with both Sens-it-iv EU Framework VI project and ECVAM (European Centre for Validation Alternative Methods).</p>
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[Link to the poster](#)

In view of Replacement

# The incorporation of 3R-alternatives in the safety evaluation of cosmetic ingredients by the SCC(NF)P

Authors	<b><u>Pauwels M. and Rogiers V.</u></b>
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Institutions/Companies	Dept. of Toxicology, Dermato-Cosmetology and Pharmacognosy Vrije Universiteit Brussel, Brussels, Belgium
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Abstract	<p><b>RESULTS</b></p> <p>Although animal tests are commonly used by regulatory bodies as the basis for hazard and safety assessments of all types of chemical substances, the upcoming testing and marketing ban in the cosmetic field legally imposes urgent substitution for these animal studies by replacement alternatives. At the EU level, the Scientific Committee on Consumer Products or SCCP (formerly called SCCNFP) assesses on a regular basis colourants, preservatives, UV filters, hair dyes and other specific cosmetic ingredients for which suspicion of potential toxicity exists. Therefore it was considered necessary to develop a system to objectively investigate the incorporation of 3R-alternatives in the dossiers submitted to this European scientific committee.</p> <p>To achieve this, we programmed a database in which the contents of all SCC(NF)P opinions are systematically loaded and which allows to search for the occurrence of alternative methods and to perform animal counts.</p> <p>A first set of results (on 185 opinions) reveals that, when validated, 3R-alternative methods appear to smoothly find their way in the submissions to the SCC(NF)P. Examples are the gradual incorporation of the fixed dose procedure, the toxic class method and the up and down procedure in the field of acute toxicity, the introduction of reduction and refinement measures in existing animal protocols in the areas of skin and eye irritation, the transition to the local lymph node assay for skin sensitisation, the standard use of the <i>in vitro</i> mutagenicity/genotoxicity testing battery and the regular occurrence of the 3T3 neutral red uptake phototoxicity test. A subsequent post-validation study on the available data sets, however, showed that several methods still required optimization and further development (e.g. mutagenicity/genotoxicity, skin irritation).</p> <p>On these 185 opinions, the database was used to compute the numbers of animals that were involved in the data generation for the dossiers submitted to the SCC(NF)P. In a worst case calculation, 21,000 animals are estimated to be used per year.</p> <p>The database will now be systematically updated with the most recent SCCP opinions. It is designed to be a practical and objective support in the further development, implementation and regulatory acceptance process of alternatives in the safety assessment of cosmetics, by :</p> <ul style="list-style-type: none"><li>- generating lists of reference compounds known to be accompanied by good <i>in vivo</i> data for a specific endpoint (e.g. optimization of human reconstructed skin models for skin irritation testing );</li><li>- calculating animal numbers related to the safety assessment of cosmetics and the evolution of these numbers over time;</li><li>- providing objective data for post-validation comparison of traditional <i>in vivo</i> data versus newly generated 3R-alternative results (e.g. Local Lymph Node Assay results versus Magnusson Kligmann Guinea Pig Maximization Test data);</li><li>- presenting a detailed overview of the incorporation of valid and/or validated alternatives in the cosmetic regulatory environment;</li><li>- supplementing existing databases (resulting from EU projects, e.g. EDETOX database on dermal absorption) with additional data from the SCC(NF)P opinions;</li><li>- delivering relevant information to support the implementation of new</li></ul>
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concepts in risk assessment of cosmetics (e.g. check of validity of TTC concept).

Since in this way the knowledge and experience of a group of European scientific experts is translated into a structured and objective tool, we believe that the cosmetic safety database has the potential to support and in some cases even speed up the highly needed regulatory trust and acceptance in 3R-alternative methods.

[Link to the poster](#)

**Information on application of 3Rs**

## The COLIPA Strategy towards animal-free Genotoxicity testing

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Abstract	<p>COLIPA is the European Trade Association representing the interests of the cosmetic, toiletry and perfumery industry. COLIPA's membership consists of: 25 national associations (representing over 2000 small &amp; medium enterprises), 20 major international companies and 5 associated or corresponding members. Due to increasing public concern and the adoption of the 7<sup>th</sup> Amendment to the Cosmetic Directive, COLIPA is funding a research and method evaluation programme to replace the need for animal testing. This poster presents the activities that the COLIPA Project Team Genotoxicity has started for improving, developing and pre-validating <i>in vitro</i> alternatives for genotoxicity testing.</p> <p>Despite the fact that hazard identification in the area of genetic toxicology uses a large number of animals, it has had less focus from an animal alternatives standpoint because sensitive <i>in vitro</i> assays already exist. However, it is becoming increasingly clear that the required battery of these sensitive <i>in vitro</i> genotoxicity assays has a low specificity (i.e. high percentage of false positive results for non-carcinogens). This creates the need for unnecessary <i>in vivo</i> follow up testing. To address this problem, the EU Cosmetics Association's (COLIPA) Genotoxicity Project Team has started a major program to develop approaches for eliminating/reducing animal testing for genotoxicity.</p> <p>Amongst other aspects, the programme consists of two main projects: a two and a half year project led by member companies of the Cosmetic industry which aims at establishing and validating new methods for genotoxicity testing in reconstructed human 3D skin models and a three year project performed at Covance Laboratories (UK) which has the ultimate goal to optimise current standard assays in order to improve specificity and thus avoid <i>in vivo</i> follow-up testing. In addition to this programme, members of the COLIPA project team are actively involved in other expert groups addressing this problem, e.g. ILSI, IWGT, ECVAM.</p>

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In view of Replacement

## OSIRIS: Integrated testing strategies for risk assessment of chemicals under REACH

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Abstract	<p><b>REACH</b></p> <p>REACH, the new European legislation on chemicals and their safe use (Registration, Evaluation, Authorisation and Restriction of Chemicals), aims at greater responsibility on industry to manage the risks that chemicals, manufactured and used in the EU, may pose to health and the environment. The safety information provided accordingly will be passed down the supply chain. By 2018, all industrial chemicals produced or imported in quantities above 1 tonne/year have to be evaluated and classified with respect to their ecotoxicological and toxicological effects. At registration, a Chemical Safety Assessment is required for tonnages above 10 tonnes per year. Although data sharing is mandatory, this procedure will result in a significant increase in animal tests in the next 10 years. However, another important aim of REACH is the reduction of animal testing where possible.</p> <p><b>OSIRIS</b></p> <p>The Integrated Project OSIRIS (Optimised Strategies for Risk Assessment of Industrial Chemicals through Integration of Non-Test and Test Information) aims to develop integrated testing strategies for REACH, considering both non-test and test information. OSIRIS is an <b>international collaborative project</b>: 31 partners from 14 European countries, including 24 research institutes, 5 small and medium-sized enterprises and 2 manufacturers of chemicals and chemical products, work together on the development of a web tool which will be made available to end-users from industry and regulatory authorities. OSIRIS integrates a large <b>variety of scientific disciplines</b> such as biology, chemistry, toxicology, ecotoxicology, toxicogenomics, statistics, information science, decision theory, as well as social sciences and economy.</p> <p><b>IMPACT OF OSIRIS FOR ANIMAL WELFARE AND THE 3R's</b></p> <p>The OSIRIS project contributes to <b>all the 3R's: replacement, reduction and refinement</b>. Integrated testing strategies (ITS) shift risk assessment from a "box-ticking" approach with extensive animal testing to a more efficient, context-specific and substance-tailored approach. The underlying principle is to take advantage of existing information before experimental testing, to group information about similar substances and to integrate exposure considerations. Thus, the framework envisaged in OSIRIS comprises complementary approaches including <b>alternative methods</b> such as qualitative and quantitative structure-activity relationships (QSARs), chemical and biological read-across and data from <i>in vitro</i> tests, as well as thresholds of toxicological concern and exposure-based <b>waiving</b>. QSARs can also be used for screening in order to prioritise chemicals and to guide and focus subsequent testing. In addition, alternatives to animal tests will be reviewed in the course of the project. Moreover, information from existing</p>
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tests is collected from a variety of sources in databases, and the quality of these data assessed. Based on the collated data, **optimisation** of existing *in vivo* and *in vitro* testing procedures will be proposed. Furthermore, a major challenge is to identify, reduce and manage the level of uncertainty in hazard assessment.

### **INTEGRATION WITH OTHER EU PROJECTS/PROGRAMMES**

In order to synchronise collection efforts of biological data, integration with other ongoing EU-funded and national projects such as CAESAR, ReProTect, SENS-IT-IV, CASCADE and the ISSCAN database on chemical mutagens and carcinogens project has been established. Through the project partner JRC (Joint Research Centre of the European Commission) additional interaction on non-testing methods takes place with key EU stakeholders.

### **ACKNOWLEDGEMENTS**

OSIRIS is an EU 6<sup>th</sup> Framework Integrated Project (contract no. GOCE-ET-2007-037017).

[Link to the poster](#)

**Replacement, Reduction, Refinement**

## Implementation of in silico models in the cosmetic industry to face the 7th Amendment of the European Cosmetics Directive

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Abstract	<p>The 7th Amendment to the EU Cosmetics Directive has made in vitro and in silico approaches to warrantee the safety of of products a key issue to support regulatory dossiers and future innovation. A ban on animal testing for chemicals to be used in cosmetics comes into effect in the EU in March 2009 for acute toxicity, genotoxicity, and skin or eye irritation. For repeat-dose (or systemic exposure) toxicity, the EU ban is subject to the 2013 deadline.</p> <p>Over the past years there have been considerable efforts to develop alternative methods, including in silico methods that would comply with regulatory constraints (OECD principles). In this context, transparent and mechanism-based approaches are needed. Progress in the area of computer science and information technology has triggered the creation of curated databases and the development of predictive models for various toxicological endpoints.</p> <p>A number of commercial or free software integrate global models targeting the 2009 endpoints. From the cosmetic industry prospective, the predictive performance of such models has to be evaluated on chemical series of interest for:</p> <ul style="list-style-type: none"><li>• Chemical prioritization</li><li>• Mechanistic understanding</li><li>• Elaboration of regulatory dossiers by providing additional information</li></ul> <p>The purpose of the present study was to use data generated on “real-life” chemicals from the cosmetic industry to challenge some of the global models available today.</p> <p>Historical in-house data obtained from the in vivo skin irritation, bacterial mutagenicity and in vivo oral acute toxicity tests, have been curated in collaboration with in-house experts for this purpose. Global models available in the Derek, MC4PC and TIMES software were evaluated for mutagenicity (Derek, MC4PC and TIMES), acute toxicity (Derek, MC4PC) and skin irritation (only Derek since ToxTree will be evaluated in the next phase of this project). Applicability domains and predictive performance were compared in order to identify potential gaps and assess the complementarities of these tools and their relevance in Integrated Testing Strategy (ITS) workflows.</p>

[Link to the poster](#)

In view of Replacement

## Spotlight on the 3Rs strategy for safety assessment: experiences of a biotech company

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Institutions/Companies	<sup>1</sup> Novozymes A/S, Denmark
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Abstract	<p><b>BACKGROUND</b></p> <p>In the changing international regulatory landscape there is an increasing pressure for developing <i>in vitro</i> alternatives. The requirements in EU's 7<sup>th</sup> Amendment to the Cosmetics Directive demand that selected safety data for cosmetic ingredients are generated using only non-animal tests and the EU chemical regulation, REACH, will also be based on non-animal methods as far as possible to establish the hazard associated with existing and new chemicals. This puts pressure on the way the industry can operate in safety testing of new products. In Novozymes A/S, we have concluded that we cannot tackle this challenge alone and therefore our strategy is to network to be able to meet our own and our customers' needs for new "non-animal tested" products and to meet the requirements from the various authorities.</p> <p><b>MAIN OBJECTIVES</b></p> <ul style="list-style-type: none"> <li>• We will continuously pursue the principles of the Three R's: Replacement, Reduction and Refinement in the development and approval of our new products.</li> <li>• We will contribute to development so that Novozymes in the future can continue to deliver innovation and new products that lives up to the requirements for safe products but without the extended use of animals.</li> <li>• We will maintain and improve the existing in-house <i>in vitro</i> tests.</li> <li>• We will continue the work on gaining acceptance by authorities and customers of <i>in vitro</i> data, especially the skin and eye irritation and skin sensitization data on enzymes.</li> <li>• We will participate actively in relevant fora promoting the development and acceptance of <i>in vitro</i> alternatives.</li> </ul> <p>By pursuing the Three R's: Replacement, Reduction and Refinement, as much as possible, we are committed to minimize the use of animals in research and safety assessments. We recognize that Replacement is a long term objective and therefore we pursue Reduction and Refinement with the same intensity.</p> <p><b>STRATEGY</b></p> <p>The principal strategy is networking, primarily within industry but also with academia and the European, global and national research programmes. We participate in meetings and fora related to <i>in vitro</i> techniques, e.g. by being member of the European Partnership for Alternatives to Animal Testing (EPAA) and IVTIP (the <i>In Vitro</i> Testing Industrial Platform).</p> <p>We participate actively in several EU funded projects, e.g. the two integrated projects Sens-it-iv, Carcinogenomics and the strategic targeted research project Nabinms. Recently, a collaboration across the <i>in vitro</i> projects funded by the European Commission (ALTER_NET) has been initiated. We participate in relevant ECVAM task force and expert meetings, in ECOPA (European Consensus Platform for Alternatives) meetings and in national, Danish, <i>in vitro</i> activities.</p> <p>Further, we continue our in-house work on integrating new assays step by step in selected areas relevant for our industry, using <i>in vitro</i> techniques accepted or proposed as valid alternatives.</p> <p>We challenge the <i>in vivo</i> toxicological programs on a case by case basis to implement the 3Rs principles as much as possible. Animal welfare is high on the agenda and the company has a dedicated internal Laboratory Animal</p>
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Review Committee.

Doing the above, Novozymes actively contributes to the dissemination of progress and knowledge, and in transfer of technologies related to the Three Rs.

### **ACHIEVEMENTS**

It is highly important to foster collaboration between industry, academia and regulators in support of a more rapid progress in the acceptance of *in vitro* models and strategies and we believe that our networking efforts help to accomplish these objectives. No institution can do this alone – networking, collaboration and communication is the only way forward.

[Link to the poster](#)

**Replacement, Reduction, Refinement**

## Progress towards allergen testing without the use of animals

Authors **Roggen E.L.<sup>1</sup>, Weltzien H.-U.<sup>2</sup>, Hermans W.<sup>3</sup>**

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Abstract	<h3>THE DRIVERS FOR NON-ANIMAL ALLERGEN TESTING</h3> <p>Massive resources are being invested by the European Commission and private industry to develop non-animal based methods that will allow identification of substances that potentially cause allergy (allergens). An urgent need to develop regulatory accepted alternative methods arises from new legislations, such as the REACH legislation on chemicals and the 7<sup>th</sup> Amendment to the Cosmetics Directive.</p> <p>These considerations prompted 28 groups from academia and industry, as well as special interest organizations and ECVAM, to establish the <i>Sens-it-iv</i> consortium (<a href="http://www.sens-it-iv.eu">www.sens-it-iv.eu</a>). The consortium aims to develop non-animal testing strategies to assess allergenic potential of skin and respiratory sensitizers as part of safety assessment strategies. It thereby clearly addresses the 3R objectives of reduction and, most importantly, replacement.</p> <h3>THE SCIENTIFIC CHALLENGE</h3> <p>The scientific approach of the <i>Sens-it-iv</i> project is to build on and exploit our increasing understanding of the mechanisms driving sensitization. This involves an improved appreciation of the biological processes that occur when tissue is exposed to sensitizing materials and to compare this with molecular indicators on the cells involved in these reactions. This work is carried out in workpackages (WP), which are grouped in a Science Module. The knowledge generated in the Science Module will be used in a Technology Module to develop assay systems that model sensitization, rather than irritation and toxicity of chemicals and proteins.</p> <h3>SCIENTIFIC ACHIEVEMENTS</h3> <p><b>Proper test compounds were identified.</b> A database has been established compiling information on well characterized skin and respiratory sensitizers with a wide range of potencies.</p> <p><b>We learned about <i>in vivo</i>.</b> To acquire a good understanding of the <i>in vivo</i> players and mechanisms involved in lung sensitisation, use is made of the precision cut lung slices (PCLS) technology. The work on mouse PCLS revealed promising differences in cytokine regulation when the slices were exposed to either sensitizers or irritants. This work has led to a standard operation procedure which was refined and implemented on human lung tissue.</p> <p><b><i>In vivo</i>-like epithelial cells (EC) were identified.</b> A catalogue of available primary EC and EC lines, as well as a catalogue with factors affecting <i>in vitro</i> cell phenotype and functionality was finalized. While for the lung a final candidate was not yet identified, skin work is now focusing on primary human keratinocytes.</p> <p><b>The most <i>in vivo</i>-like dendritic cells (DC) were identified.</b> In analogy with the work performed on EC, it was attempted to find the most <i>in vivo</i>-like DC.</p>
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A large number of DC lines were catalogued. As yet, the DC line showing the most *in vivo*-like phenotype and functionality is the MUTZ-3 cell line. Its various differentiation states appear to resemble different DC states *in vivo*.

**Description of *in vitro* conditions supporting the most *in vivo*-like EC-DC interactions is in the final phase.** An allergic response involves a lot of interactions (cross-talk) between EC and DC. Principally three different systems in which EC and DC are co-cultured have been used to address EC-DC cross-talk. Most of these studies still are in a very experimental phase, but promising results were obtained for the skin.

**Identification of *in vivo* EC and DC markers with relevance for *in vitro* assessment is rapidly progressing.** A concerted action involving WP4 (Genomics), WP5 (Proteomics) and WP6 (Metabonomics) is collecting useful marker profiles describing sensitization and potency.

**The study of DC-T-cell interactions has identified new opportunities.** Evidence has been provided that there may be a role of innate immune responses in allergy development. This may lead to the detection of new marker molecules to discriminate sensitizers from non-sensitizers.

**The impact of sensitizers on cellular protein profiles is studied.** It is important to not only assess the genomic reaction of a cell to chemicals, but also to study the protein profile following exposure to a chemical (proteomics) and to identify proteins which preferentially interact with allergenic chemicals (haptens). Proteome analyses in allergen-treated primary keratinocytes and monocyte-derived DC revealed numerous, partially cell-specific hapten-binders.

**The understanding of the role of metabolism in chemical sensitization is growing.** Many allergenic compounds lack chemical reactivity towards proteins, but require prior enzymatic transformation into reactive metabolites. A combined bio-analytical and cellular immunological platform was established to determine how such chemicals interact with immune cells (both antigen presenting cells and T-cells), and to relate metabolism, compound distribution and covalent protein-binding to regulation of transcription and activation of immune cells.

## TECHNOLOGICAL ACHIEVEMENTS

**The acquired data are properly managed.** A central repository for *Sens-it-iv* data was implemented. The basic infrastructure includes a control system, that allows tracking the progress of the project and to restore previous stages of the project. A web interface allows for the submission and retrieval of *Sens-it-iv* data.

**The *in vitro* assay is being evaluated.** Preliminary experiments using a test panel of chemicals including 4 sensitizers and 3 non-sensitizers showed that MUTZ-3 DC and some myeloid cell lines increased expression of surface marker CD86 and cytokine IL-8 after exposure to sensitizers but not after exposure to non-sensitizers. This test model is currently under evaluation.

## ACKNOWLEDGEMENTS

This work was supported in part by a grant from the European Commission as part of the Integrated project ' Novel Testing Strategies for In Vitro Assessment of Allergens (*Sens-it-iv*), LSHB-CT-2005-018681'

[Link to the poster](#)

In view of Replacement

# The applicability of non-animal safety assessments: experiences of the In Vitro Testing Industrial Platform IVTIP

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Abstract	<p><b>WHO OR WHAT IS IVTIP?</b></p> <p>The <i>In Vitro</i> Testing Industrial Platform (IVTIP) is an association composed of European companies with an active interest in <i>in vitro</i> testing, not only to be used in regulatory safety testing but also for compound discovery and product development. Member companies represent the pharmaceutical, chemical and cosmetic sector, as well as independent contract research organizations. The members of IVTIP are all active in supporting and applying the principle of the 3Rs (Replacement, Reduction and Refinement of animal testing), and in promoting the adoption of the fourth R (Responsibility in research promoted by industry). IVTIP is a science-driven organization existing for and by the members.</p> <p><b>WHAT IS IVTIP DOING?</b></p> <p>The representatives in IVTIP are scientists who are active in the area of <i>in vitro</i> and/or <i>in silico</i> methods. IVTIP provides advice to European Union (EU) Institutions about industrial activities and needs for research, development and application of alternatives to animal testing. Vice versa, they inform industry about upcoming EU activities and new regulations involving <i>in vitro</i> testing. IVTIP is an active group of scientists, who discuss new opportunities, inform each other about promising developments and participate in EU projects. The liaison with academic groups is very important to stimulate the applicability of techniques and methods for industrial use, thereby ensuring effective dissemination through transfer of both technology and knowledge.</p> <p><b>WHAT ARE THE DEMANDS OF INDUSTRY REGARDING <i>IN VITRO</i> / <i>IN SILICO</i> TESTING?</b></p> <p>For the industry, it is important that the results obtained with <i>in vitro</i> methods are relevant and predictive to <i>humans</i>. Results should be reliable, reproducible, unequivocal, relatively simple, robust, cost effective and regulatory acceptable. Regulatory accepted tests would be preferred, however since the current process of validation is very time consuming, it is acceptable if the validity of the assay and applicability for certain compound classes can be demonstrated.</p> <p><b>WHEN ARE <i>IN VITRO</i> / <i>IN SILICO</i> TESTS USED BY THE INDUSTRY?</b></p> <p><i>In vitro</i> and <i>in silico</i> testing are used for safety assessment, as well as discovery and development of new compounds and products. Not only internationally validated and regulatory accepted assays (for authorities) are used, but also many different tests that are used and validated on a smaller scale in-house, investigating different end points (sometimes industry specific) using different combinations of cells, tissues and compounds. The</p>
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*in vitro/in silico* tests are used to assess biological activity, structural alerts, working mechanisms, and focus on making go/no-go decisions regarding further development or maintenance of compounds. Examples of computer based tools and integration of *in vitro* methods will be presented.

### WHAT WILL THE CHALLENGES BE FOR THE FUTURE?

- The quality of *in vitro/in silico* data should be improved (reduce false positives), e.g. by using human tissue or target organ specific models.
- The *in silico*, *in vitro*, *in vivo* and human data should be integrated to increase the predictivity and improve the extrapolation to the human situation. Integrated testing will be challenging because of its complexity, but seems the way to go. Single replacement is usually not possible.
- The integrated *in vitro/in silico* data have to be translated into an *in vivo* message useful for risk assessment and risk management. A major challenge is the definition of no effect levels (NOELs) using *in vitro/in silico* data.
- The regulatory bodies should be involved in early development of new methods to increase the possibilities for successful implementation and acceptance. In the light of the new EU legislation, such as REACH and 7<sup>th</sup> Amendment of the Cosmetic Directive, development and specifically validation and acceptance of alternative methods should be faster to meet the requirements of these new regulations.
- New ways of risk evaluation are needed, including risk communication, risk management and risk perception of the general public.

[Link to the poster](#)

In view of Replacement

## ECOPA , a European umbrella organisation of national consensus platforms to foster research and promote alternatives

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Abstract

**RESULTS**

*ecopa*, the umbrella organisation of national consensus platforms for 3R-alternatives at the EU level, supports *epaa* in its co-chair, mirror and working groups functions. It also organizes the bi-annual research meeting eSI, i.e. the *ecopa* Science Initiative, in October 2008 and this for the third time. The goal is to bring renown senior scientists into contact with young researchers in order to foster research in 3R-alternative methods, and initiate new avenues of research in the area. Thereby, it is also intended to "recruit" a younger collective of scientists carrying the process of 3R-implementation further. The programs of the past eSI meetings are exhibited on the website, [www.ecopa.eu](http://www.ecopa.eu), solely devoted to sound science and novel approaches. Topics for future activities are highly welcome. In addition, actual issues and aspects in the legal, political and R+D funding arena are covered in *ecopa's* Annual Workshop (the next one takes place on November 29 and 30). Topics are including *ecopa's* involvement in 3R-alternative method projects in the EU R+D Framework Programmes, the most recent being START UP run by *ecopa* itself as the coordinator. It addresses R+D bottlenecks in the pharmaceutical industry. After three expert meetings in 2008, this project will cover the 3Rs in three open workshops in 2009, each of them devoted to 1 R. This is the way forward to arrive at the next level of research in this area. Beyond that, *ecopa* with its annual workshop as a forum for all stakeholders in this area, regular newsletters (*ecopa* messenger), updated website and pan-European and national activities is THE dissemination platform for 3R-alternative methods in all its aspects in Europe.

**ACKNOWLEDGEMENTS**

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[Link to the poster](#)

**Replacement, Reduction, Refinement**

# The zebrafish embryo: an alternative model to screen for developmental neurotoxicity and teratogenicity of compounds

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Abstract	<b>INTRODUCTION</b> <p>The developing human system is susceptible to many toxicants, and chemical exposure during pre- and postnatal development may cause lasting deficits. Such damage can range from subtle to severe, and it may impose substantial burdens on affected individuals, their families, and society. Testing compounds for developmental toxicity endpoints is an important societal and scientific goal. Current international guidelines for developmental toxicity and neurotoxicity (DNT) involve exposure of pregnant animals, mostly rats and rabbits, and subsequent evaluation of effects in their progeny, having their economical and ethical drawbacks. According to the EU “White Book, Strategy for Future Chemicals Policy” <i>in vitro</i> data need to be collected for all chemicals with a production volume &gt; 1 ton per year (REACH). The implementation of the White Paper may result in some 30,000 chemicals requiring further toxicity testing before 2012. The estimated testing cost for developmental toxicity testing is estimated to be the highest (31% of total costs ≈ 470 M€, Pederson <i>et al.</i>, 2003). Moreover the number of animals needed for developmental toxicity screening is estimated to be 2,192,794 (13.2% of total animals needed) (‘The impact of REACH’: The report of the CONAM/ecopa Chemical Policy Working Group, 2007, www.ecopa.eu). The development of novel alternatives for developmental toxicity including developmental neurotoxicity and teratogenicity, in order to reduce animal experiments is therefore gaining great interest.</p> <p>Three types of alternative mammalian methods (cell cultures, organ cultures and embryo cultures) have already been considered in (pre-)validation studies. Although these methods can be useful in mechanistic studies, none of them can cover all the aspects of prenatal development. In order to study complex toxicological mechanisms related to (neuro-)development, the whole organism approach might offer advantages. Both for ethical reasons, and practical considerations, the zebrafish embryo does offer numerous opportunities to be explored as an alternative vertebrate model. A short term zebrafish assay might give an ethically acceptable, and cost-efficient test for hazard assessment of numerous chemicals.</p> <b>OBJECTIVES</b> <p>The overall purpose of this study is to optimize and evaluate methods with zebrafish embryos, as a fast model system for high-throughput screening of drugs and chemicals with potential effects during early development. The planned experiments will characterize early malformations and locomotor dysfunction caused by particular test substances, respectively teratogenic and developmental neurotoxic compounds.</p> <b>ACHIEVEMENTS</b> <p>Known teratogenic and developmental neurotoxic compounds have been selected and they are evaluated using newly-developed in-house standard protocols.</p>

The teratogenic assay includes the time-related evaluation of morphological endpoints like heart beat, tail detachment, formation of somites, otoliths, eyes, spinal cord, ... at embryonic and larval stages up to 144 hours post fertilization (hpf). For this test, a test compound is solved in water in which the embryos are placed. Afterwards, microscopic observations are made and the extent of malformations, compared to lethality, that is observed in the embryos or larvae at different concentrations of the test compound, will give an indication of the specific teratogenic potency of a chemical. (Selderslaghs *et al.*, manuscript submitted)

For the neurotoxic assay, locomotor activity of the zebrafish embryos and larvae is selected as a measure for neurobehavioral disorders. Zebrafish embryos are exposed to a test compound in water and tail coilings in embryos (age 24 hours) and swimming behavior of larvae (age 120-144 hours) are studied. To improve fast and objective data collection, movie files are created which are then analyzed with the appropriate software packages. For the embryos, the number and duration of tail coilings are determined, followed by the evaluation of differences between the data for controls and exposed embryos. For the larvae velocity, distance moved and total movement are determined. Based on these parameters, the impact of the test compound on the behavior of the zebrafish larvae is evaluated.

The procedures, ascribed above, are currently applied for a number of compounds (retinoic acid, caffeine, valproic acid, ethanol, chlorpyrifos, acrylamide, lead, ...) and results do allow ranking of chemicals according to their potency.

## CONCLUSIONS

The results obtained with the above described methods do allow to classify chemicals either as developmental toxicants, as neurotoxicants or as negative for test endpoints. Further studies with an extended panel of chemicals (up to 20), will be used to develop a prediction model for mammalian teratogenic and neurodevelopmental effects. The validity of the zebrafish test for the screening of chemicals is demonstrated after evaluation through comparison with available mammalian *in vivo* and *in vitro* data. The (pre-)validation of these methods is urgent and of interest to industry as they can be used for screening purposes of both chemical as pharmaceutical products.

Furthermore, in light of the 3R's, these methods have the potential to (partly) replace and thus reduce animal testing with mammals. Refinement is also taken into account since exposure of the embryos occurs in a high-throughput manner, within a few days after fertilization and in a low test-volume.

## ACKNOWLEDGEMENTS

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[Link to the poster](#)

Reduction, Refinement

## CEIISens-Eco8: Development of a strategy to replace acute fish toxicity tests

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Abstract	<p><b>BACKGROUND TO ACUTE TOXICITY TESTS WITH FISH</b></p> <p>The EU guideline REACH implies testing of all chemicals which are produced or imported into EU above a ton per year with regard to their potential risk to humans and environment. The acute fish toxicity test (OECD guideline 203, [1]) is today the most commonly applied vertebrate test in environmental risk assessment. Within this test system a large number of fish are required. At least 5 different concentrations of the test chemical with at least 10 fish per concentration are needed. The death of the fish is scored after 96h of exposure and the concentration calculated which kills 50% of the fish. Due to REACH, the number of fish needed for ecotoxicological testing is estimated to increase by another 4.4 million [2], which emphasizes the need for appropriate alternative methods.</p> <p><b>OBJECTIVES OF THE CEIISens-PROJECT</b></p> <p>A replacement of the acute fish toxicity test by alternative methods has long been sought. In recent years, significant progress has been made with the development of the <i>Danio rerio</i> (zebrafish) embryo toxicity test (<i>DaT</i>, [3]) as a potential alternative. The use of cell lines, especially from fish, is another promising alternative. But until today, cell lines appear to be at least one order of magnitude less sensitive than the whole fish [4]. Finally, mechanism-based cell line and embryo data can inform computer models to advance Quantitative-Structure-Activity Relationships.</p> <p>The transatlantic project CEIISens-Eco8 aims to improve the fish embryo test and the cell line approach to achieve international acceptance as a replacement for the acute fish toxicity test. The following steps are proposed: 1) modification of the culture environment, 2) selection of mode-of-toxic action specific endpoints, 3) consideration of bioavailable versus nominally added chemical concentration and 4) the capability-driven as opposed to random selection of cell lines. These four steps shall aid in the development of a more rational, mechanism-based alternative testing strategy.</p> <p>The first step of the project was the development of a systematically derived list of representative chemicals [5]. To avoid the need for additional fish toxicity tests, we used the U.S. EPA Duluth fathead minnow database for fish acute toxicity values. For cell toxicity values we consulted the Halle Registry of Cytotoxicity. The aim was to select chemicals with a wide range of physico-chemical properties (e.g. lipophilicity and volatility), as well as different modes of toxic action. The selection procedure was mainly based on simple correlations of 1) fish and cell toxicity values and of 2) the physico-chemical parameters.</p>

Further, outliers were picked. Outliers are chemicals whose fish and cell values are far apart. The cause of these differences is particularly important to understand and to avoid the future creation of outliers with alternative testing protocols. The CEII Sens list of chemicals is now used for data acquisition in cell lines and embryos and for the development of predictive Quantitative-Structure-Activity Relationships [6].

As mentioned before, cells seem to be less sensitive as the whole fish. The next steps are therefore aimed at the identification of the causes. First tests showed that the set-up of the cell toxicity assay and physico-chemical properties of the chemicals are the most limiting factors. There is a strong difference in the toxicity values depending on the used solvent, solvent concentration as well as the dosing method applied. The differences between the test conditions were greatest for 1,2-Dichlorobenzene, a rather lipophilic and volatile compound. For the water-soluble and non-volatile compound Sodium dodecyl sulfate no significant differences between the different test conditions could be seen. Determination of bioavailable chemical concentrations shall now shed light on the relationship between physico-chemical properties of the chemicals and the toxicity observed.

CEII Sens will give mechanistic insight into the question why differences between the fish test and alternative approaches (cells, embryo) exist and how they can be overcome. The *DarT* for example has been implemented in the German national regulatory testing of waste water in 2005 by replacing the acute fish toxicity test. Currently this test is under consideration to become an OECD guideline for replacement of the acute fish toxicity test. But there is one major drawback of the *DarT*: it is still necessary to maintain fish in aquariums. Though only the freshly fertilized eggs are used for the test, which are considered as non-protected developmental stage. With the use of permanent fish cell lines no fish facilities and fish maintenance are needed and quantitative measures of toxicity can more easily and effectively be obtained. There are several ways how the developed tests can be applied: 1) in a rough pre-screening for the toxicity of chemicals or waste water and therefore help to reduce the number of acute fish toxicity tests or 2) as a complete replacement of the acute fish toxicity test in environmental risk assessment. The advantage of both CEII Sens methods is, that they can be applied as high through-put methods, meaning it is possible to analyse more samples in less time (24 or 48h) compared to the fish test (96h).

#### **ACKNOWLEDGEMENTS**

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[Link to the poster](#)

**Reduction in view of replacement**

## Reporting 3Rs parameters in European scientific research papers-are we making progress?

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Abstract	<p>It is 20 years since both the Council of Europe Convention ETS123 and the EU Directive 86/609 were introduced, recommending the implementation of the 3Rs (reduction, replacement and refinement) in animal experiments and providing guidance on animal housing. It might therefore be expected that this might have influenced reports of 3Rs related parameters in animal research papers over time.</p> <p>In order to test this hypothesis, a literature survey of animal-based research reported in 15 key journals was conducted. A randomly selected sample of original research papers arising from European institutions that involved primates or genetically modified mice were identified for the years 1985-6 and 2005-6 (N=50 each). Each paper was reviewed for reports of 10 parameters corresponding to replacement (consideration of non-animal methods), reduction (justification of number of animals required) and refinement (housing –related; size, social, object and food enrichment and procedure-related; the use of training, humane endpoints, anaesthetic and analgesia).</p> <p>Despite increasing reports of adherence to the legislation and codes of practice, reports of reduction and replacement considerations were extremely low (0-6%) with no significant difference between year or species. Reports of refinement related improvements were higher but still low (0-45%) with only a few significant differences between year or species. With increasing provision of supplementary information in online papers it should be more, rather than less, possible to acknowledge 3Rs related parameters within research papers. This is an important issue to be tackled by academics and journal editors if the 3Rs is to be promoted nationally and internationally.</p>

[Link to the poster](#)

**Information on application of 3Rs**

## The Dutch-Belgian Society for In Vitro Methods (INVITROM)

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Abstract	<p>Scientific advancement is relying on experimental work as well on discussions of the hypotheses, the methodologies and the results used in scientific research. In biomedical sciences a significant part of the work is performed with methods using animals as model systems. Undoubtedly, these models have greatly advanced our knowledge of biological systems in many areas, ranging from fundamental insights in natural processes to the applications in medicine, pharmaceuticals and also in the safe use of chemicals. However, there are drawbacks and limitations to these models. From the ethical point of view, criticism is increasing on the use of animal models and the distress that is causing this to the animals. This has prompted the drive for the three Rs: refinement, reduction and replacement. Also from a scientific point of view, the use of other systems than intact animals has shown to be preferable. It is in the area of the development and the use of in vitro methods, such as cell and tissue culture systems, that the Dutch-Belgian Society for In Vitro Methods (INVITROM) intends to be a platform for discussion, enabling the advancement of these methods in the wider field of biomedical and biological research. INVITROM aims to do this by promoting the development of such methods, their use and their acceptance by the scientific world as well as by regulatory bodies. INVITROM will do this by acting as a platform for discussions, contacts, and co-operation, by organising working groups, symposia, an extensive network of experts, an e-mail list and a web site. We consider these activities of very high importance, also in the light of recent and future developments in European legislation, e.g. REACH and in the framework of sustainable enterprise.</p> <p>INVITROM currently hosts working groups in the following areas:</p> <ul style="list-style-type: none"><li>• <i>In vitro disease models</i></li><li>• <i>The use of animal sera in cell and organ cultures</i></li><li>• <i>Slice methods and tissue preservation</i></li><li>• <i>Human material in research</i></li><li>• <i>Mechanistically-based in vitro models</i></li></ul> <p>Subjects of past meetings were:</p> <ul style="list-style-type: none"><li>• <i>"Applications of human tissues in in vitro research", 15 March 2007, Oss, The Netherlands</i></li><li>• <i>"Towards Better Reliable In Vitro Methods" 22 April, 2008, Bruges, Belgium</i></li></ul> <p>The topic of March 2008 will cover stem cells and their application in the 3Rs.</p> <p><b>ACKNOWLEDGEMENTS</b></p> <ul style="list-style-type: none"><li>• Johnson &amp; Johnson Pharmaceutical Res. &amp; Dev.</li><li>• Proefdiervrij</li><li>• NOTOX</li></ul>

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**Advancement of Replacement methods**

## Development of a new strategy to stabilize liver cells in culture

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Abstract	<p><b>RESULTS</b></p> <p>During the preclinical phase of drug development, a number of questions with respect to safety and efficacy of the new molecules must be answered. For that purpose, <i>in vitro</i> methods and more specifically cultured liver cells (hepatocytes) are used. A major problem, however, is their short life span and the loss of their liver-specific functions such as drug metabolism which means that the cells loose the enzymes that convert toxic molecules to harmless substances. To overcome this problem, several methods were applied, including changes in the culture medium composition, culturing the cells with other cell types or on different matrices. None of these methods, however, was successful. Therefore, new strategies needed to be explored. Here we show that special molecules (so-called epigenetic modifiers that are able to change the chromatin structure of the cells) are innovative tools to better maintain the typical liver functions of the cells. Indeed, upon exposure to these molecules (Trichostatin A, TSA, is an example) the cell cultures can be longer kept viable, they secrete more albumin and the important drug metabolising pathways are better maintained. In addition, the expression of a number of transcription factors of major importance for the liver and drug metabolism (e.g. HNF4<math>\alpha</math>, C/EBP<math>\alpha</math>) is increased throughout the whole culture period.</p> <p>In conclusion, the method of exposing liver cells to epigenetic modifiers such as TSA makes it possible to perform better screenings of new molecules. Indeed, the results more closely reflect the <i>in vivo</i> situation. As more reliable and more relevant <i>in vitro</i> data result in less animal testing, in particular when human liver cells are involved, this innovative methodology contributes to the reduction of animal testing during drug development.</p> <p><b>ACKNOWLEDGEMENTS</b></p> <p>This work was supported by grants from the Fund for Scientific Research (FWO) Vlaanderen, Belgium; the Research Council (OZR) of the Vrije Universiteit Brussel, Belgium; and the European Union Sixth Framework Program (STREP projects no. 504761 PREDICTOMICS and no. 037499 LIINTOP). Vanhaecke T. is a postdoctoral fellow of the Fund for Scientific Research (FWO) Vlaanderen, Belgium.</p>

[Link to the poster](#)

Reduction

## Development of a transcriptomics-based *in vitro* respiratory sensitization assay using 3 human lung cell types

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Abstract	<p><b>INTRODUCTION</b></p> <p>Respiratory sensitization is a concern for occupational and environmental health in consumer product development. Despite international regulatory requirements there is not yet an established protocol for the identification of chemical respiratory sensitizers. In the development of methods for respiratory sensitization, <i>in vitro</i> approaches have been a continuous challenge. Furthermore, there is considerable interest in understanding the mechanisms through which chemical allergens induce respiratory allergy.</p> <p><b>OBJECTIVES</b></p> <p>The major goal of this study was to investigate the alterations in gene expression of 3 human lung cell lines (BEAS-2B, A549, and THP-1 cells) after exposure to respiratory sensitizers and respiratory non-sensitizing chemicals, and to identify genes that are able to discriminate between both groups of chemicals. The selected lung cell lines represent each a different cell type of the respiratory tract. BEAS-2B and A549 represent respectively bronchial epithelial and alveolar epithelial type II cells, THP-1 cells were differentiated into the macrophage-like cell type.</p> <p><b>MATERIALS AND METHODS</b></p> <p>Each cell line was exposed for respectively 6, 10, and 24 hours to the respiratory sensitizers ammonium hexachloroplatinate IV (HCpT), hexamethylene diisocyanate (HDI), and trimellitic anhydride (TMA), the irritants acrolein (ACR) and methyl salicylate (MeSA), and the skin sensitizer 1-chloro-2,4-dinitrobenzene (DNCB). Overall, changes in gene expression were evaluated using Agilent Whole Human Genome 4x44K oligonucleotide arrays. A Fisher Linear Discriminant Analysis was used on each cell line data set to obtain a ranking of genes that reflects their potential to discriminate between respiratory sensitizing and respiratory non-sensitizing chemicals. Based on the differentially expressed genes, a pathway analysis tool was used to identify possible underlying mechanisms of respiratory sensitization.</p> <p><b>RESULTS</b></p> <p>In all 3 cell models, different marker genes were obtained that can distinguish respiratory sensitizers (HCpT, HDI and TMA) from irritants (ACR, MeSA) and the skin sensitizer DNCB. Our data suggest that in BEAS-2B cells and in THP-1 macrophages respectively the canonical PTEN and PDGF signaling pathway are probably specific for respiratory sensitization. In contrast, none of the canonical signaling pathways activated in A549 cells were specific for respiratory sensitization.</p> <p><b>CONCLUSIONS</b></p> <p>These results show that the evaluated human lung cell lines may be useful human <i>in vitro</i> alternatives for further use in a test strategy towards the reduction of animal use for respiratory sensitization. However, these first analysis need to be further supported by an extended dataset for other chemicals, with priority on gene expression studies in the BEAS-2B and THP-1 cellular models.</p>

[Link to the poster](#)

Reduction

## Establishment of an *in vitro* model of liver cell death

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Abstract	<b>RESULTS</b> <p>Historically, the liver has gained particular attention by toxicologists, as it represents a key target organ of chemical-induced cell injury. Damage to hepatocytes, the most important cells in the liver, may burgeon into the onset of cell death. The latter mainly occurs <i>via</i> a well-orchestrated process called "apoptosis". A number of protocols have been described to study liver cell death <i>in vivo</i>, including the direct administration of cell death-evoking toxicants to animals and the use of genetically modified animals. Such experiments not only raise serious ethical questions, but are also of limited scientific value. Indeed, apoptotic cells are barely detectable <i>in vivo</i>, as they are rapidly removed by surrounding (non-hepatocyte) cells. Such constraints can be overcome by using <i>in vitro</i> experimentation. Our group has recently developed an <i>in vitro</i> system to study liver cell death. Basically, this system consists of primary cultures of hepatocytes that are exposed to a combination of chemicals with clear-cut cell death-eliciting properties. The model was characterized by testing a battery of well-known cell death markers. Using a number of biochemical techniques, we could demonstrate that the entire time course of apoptosis can be monitored in our experimental setting, whereby all typical apoptotic features are manifested. Based on our findings, it can be concluded that a reliable <i>in vitro</i> model of apoptotic liver cell death was developed. This model can specifically be used in early drug development to screen for effective anti-apoptotic molecules and more generally to test anti-apoptotic properties of drugs. The advantage of the model particularly lies in the fact that it replaces an <i>in vivo</i> model which, scientifically spoken, does not provide convincing results, by a robust <i>in vitro</i> alternative which also allows a clear characterization of the pathways involved. It should be considered as an additional tool to improve and enlarge the set of alternative methods available in modern drug development.</p> <b>ACKNOWLEDGEMENTS</b> <p>This work was supported by the grants from the Research Council of the Vrije Universiteit Brussel (OZR-VUB), the Fund for Scientific Research Flanders (FWO-Vlaanderen) and the European Union (FP6 projects carcinoGENOMICS and LIINTOP).</p>

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Replacement

## Go3R – the Knowledge-Based Search Engine for Information on Animal Testing Alternatives

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Abstract	<p><b>RESULTS</b></p> <p>The core of any strategy to reduce animal experiments lies in the availability of relevant information regarding alternative methods. EU Directive 86/609/EEC for the protection of laboratory animals obliges scientists to consider whether a planned animal experiment can be substituted for by another scientifically satisfactory method which is reasonably and practicably available. To meet this regulatory obligation, scientists must consult the relevant scientific literature prior to any experimental study using laboratory animals.</p> <p>The internet enables access to a huge quantity of information. Nevertheless, it is difficult and time-consuming to select adequate information from this vast amount. Moreover, at the end of a query it remains unclear if all the required relevant information actually has been retrieved. This is where a new generation of knowledge-based search technology take effect.</p> <p>In April 2008, the beta version of Go3R (<a href="http://www.Go3R.org">www.Go3R.org</a>), the first knowledge-based search engine for alternative methods in agreement with the 3Rs principle, was released. Go3R is free of charge and enables scientists and regulatory authorities involved in the planning, authorisation and performance of animal experiments to determine the availability of alternative methods in a fast, comprehensive and transparent manner. The technical basis of this search engine is a specific expert knowledge, captured within an ontology. An ontology is a network of – also hierarchically – grouped “concepts” like subject areas, indicative for the respective field of research. It specifies the unambiguous meaning of relevant terms and depicts the complex relationships existing between them. With the help of such an ontology, the content of any document can be semantically determined by the mapping of the unique pattern of concepts and terms utilised in it. An essential step in the development of Go3R has involved the creation of an appropriate ontology by defining those concepts and terms that are relevant for alternative methods in accordance with the 3Rs principle and inferring the unique relations between them. The engine can now assist searchers by pre-sorting the retrieved documents according to their respective pattern of concepts and by attributing them to delimited topics. The result is an “intelligent table of contents” representing the hit list of relevant concepts and terms used in the documents, which the searcher can then use to navigate through the “thicket of information” of his query result.</p> <p>The poster shows by an example how the use of Go3R speeds up and improves the procedure of information retrieval – making it more comprehensive and transparent. Go3R improves animal protection in accordance with the 3Rs principle by reliably revealing alternatives to animal experiments documented in the literature.</p>

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge the National German Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET) at the German Federal Institute for Risk Assessment (BfR) in Berlin and Transinsight GmbH, Dresden, for the funding of the feasibility study that lead to the creation of the Go3R.org semantic search engine prototype. Transinsight GmbH is thanked for additional funding and the provision of technology necessary for the internet presence of the Go3R search engine. The financial support of the BASF and especially Dr. Robert Landsiedel, Head of the Short Term Toxicology Department of BASF, helping to provide the service Go3R on the internet, is also highly appreciated.

[Link to the poster](#)

**Information on application of 3Rs**

# Assuring Safety without Animal Testing: New Risk Assessment Approaches for Skin Allergy and Cancer

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Abstract	<p><b>RESULTS</b></p> <p>Assuring consumer safety without the ability to generate animal test data on novel ingredients is a considerable challenge. However, through the application of new technologies and the further development of risk-based approaches for safety assessment, we remain confident it is ultimately achievable.</p> <p>Recent changes in EU legislation [i.e. 7<sup>th</sup> Amendment to the EU Cosmetics Directive; EU Chemicals Regulation (REACH)] have made developing alternative approaches to assure the safety of consumer products a key business need. A substantial research programme was therefore initiated by Unilever in 2004, aimed at critically evaluating the feasibility of a new conceptual approach [1] based upon the following key components:</p> <ol style="list-style-type: none"><li>1. Developing new risk assessment approaches</li><li>2. Developing new biological (<i>in vitro</i>) and computer-based (<i>in silico</i>) models</li><li>3. Evaluating the applicability of new technologies for generating data that can be interpreted for risk-based safety assessment (e.g. omics, informatics).</li></ol> <p>We have focussed initially on risk-based approaches for skin allergy (sensitisation), since this is an important consumer safety endpoint for home and personal care products and an endpoint where animal data (e.g. mouse local lymph node assay data) are often needed currently to perform risk assessments. A new conceptual framework for skin sensitisation risk assessment that does not require the generation of new animal data is being evaluated. We are also exploring the value of utilising new consumer exposure modelling techniques, existing <i>in vivo</i> (animal and human) data and new <i>in silico/in vitro</i> hazard characterisation approaches to inform the risk assessment framework.</p> <p>A similar rationale is being applied to develop a new risk assessment approach for cancer that does not require the generation of new animal test data. Novel biological insights are being generated that will be capable of informing the risk-based approach and we are investigating the applicability of several ‘omics’ and other analytical technologies for constructing, visualising and interrogating biological networks. By combining exposure and dose considerations together with a greater understanding of the influence of biological thresholds, we are striving to integrate <i>in vitro</i> test data into a more informed decision making process.</p> <p>These two new risk assessment approaches are part of Unilever’s ongoing effort to develop novel ways of delivering consumer safety and represent our strategy to ultimately achieve full <b>replacement</b> for both skin allergy and cancer endpoints.</p> <p><b>ACKNOWLEDGEMENTS</b></p> <p>This work has been performed by several teams within Unilever and in collaboration with the following external partners: Barts and The London School of Medicine and Dentistry, UK; BioFocus DPI, UK; BioReliance, USA; Charles River Laboratories, UK; Entelos Inc., USA; Imperial College London, UK; Information Network of Departments of Dermatology (IVDK), Germany; Kings</p>

College London, UK; Lancaster University, UK; Massachusetts Institute of Technology, USA; Oroxcell, France; ProBioGen, Germany; University of Cambridge, UK; University of Liverpool, UK; University of Manchester, UK; University of Nottingham, UK; University of Pittsburgh, USA; University of Southampton, UK

[1] Fentem, J. *et al.* (2008) AATEX **14**, 15-20.

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**In view of Replacement**

## Cefic LRI's research engagement for the 3R principles

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Abstract	<p><b>THE CEFIC LRI PROGRAMME</b></p> <p>The Long-range Research Initiative's (LRI) mission is to identify and fill gaps in our understanding of the hazards posed by chemicals and to improve the methods available for assessing the associated risks.</p> <p>LRI funds high-quality research within universities as well as public, private and governmental research institutes. Every year, Requests for Proposals (RfPs) are published. From the proposals submitted, projects are selected based on scientific merit and relevance. Projects are implemented in cooperation with the scientific community and government institutions. Researchers are required to publish results. Results are also disseminated at the annual LRI Workshop (the 10<sup>th</sup> Anniversary event takes place on 20-21 Nov 08).</p> <p><b>LRI RESEARCH PROJECTS</b></p> <p>A number of LRI projects are relevant to the 3Rs. Selected results from the following projects will be presented. Further information can be found at <a href="http://www.cefic-lri.org">www.cefic-lri.org</a>.</p> <p><b>REPLACEMENT</b></p> <p><i>Development of a strategy to predict acute fish lethality using fish cell lines and fish embryos (ECO8) and Initial explorations on alternatives for the fish chronic toxicity testing (ECO8.2):</i> The project aims at developing a strategy that allows the replacement of acute fish lethality tests with fish cell line assays and/or fish embryo tests. Existing embryo tests are being extended to predict chronic toxicity.</p> <p><i>Human neurospheres as an alternative testing system for developmental neurotoxicity (LRI Innovative Science Award 2006):</i> A novel <i>in vitro</i> system to predict developmental neurotoxicity based on human neurospheres was developed and tested using model compounds.</p> <p><b>REFINEMENT</b></p> <p><i>Model organisms with genetically sensitized signal transduction pathways as predictors for mammalian developmental toxicity (AIMT1):</i> The overall objective of the research is to determine whether model organisms with genetically sensitized signal transduction pathways can be used as predictors of mammalian developmental toxicity.</p> <p><i>Rapid Generation and Analysis of Physiologically-Based Pharmacokinetic (PBPK) Models (B3.7):</i> Modelling software called MEGen (Model Equation Generator) for the rapid generation and analysis of PBPK models was developed.</p> <p><b>REDUCTION</b></p> <p><i>AMBIT software for data management (EEM9):</i> An open-source software was developed to support the application of QSAR models and other chemoinformatic tools in data-gap filling (read-across) and grouping of chemicals (categorisation).</p> <p><b>ACKNOWLEDGEMENTS</b></p> <p>We would like to thank all researchers working on LRI projects as well as the industry experts serving as project monitors. The Cefic LRI Programme is sponsored through the Cefic membership fee and fully financed by the European chemical industry.</p>

[Link to the poster](#)

**Replacement, Reduction, Refinement**