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Alternative testing – The intelligent way to REACH compliance

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Abstract
On June 1, 2007 the new European chemical regulation REACH entered into force. One of the key principles of REACH requires the performance of new vertebrate studies only as a last resort and to limit the duplication of other studies. A variety of of data generation measured including the use of existing human and animal data, read-across, quantitative structure activity relationship modelling and alternative in vitro test methods are specified in the guidance. Thus intelligent testing strategies are based on optimal use of all data generation sources and measures available. The presentation will focus on perspectives and challenges, concentrating on the application of alternative in vitro test methods to predict the most important end points of human health toxicity under consideration of available resources, forseen in the regulatory context, and in compliance with common moral and ethical values. RCC appreciates the challenges in toxicology, is proactively including integrated testing strategies in the safety evalution of chemical compounds and is supporting with scientific knowledge and expertise.

Keywords: *in vitro* methods, integrated testing strategies, intelligent testing, REACH regulation

On June 1, 2007 the new European chemical regulation REACH will enter into force. One of the key principles of REACH requires to perform new vertebrate studies only as a last resort and to limit the duplication of other studies. Under Annex XI a set of waiving options is introduced. This allows to completely refrain from filling data gaps if justified by scientific or exposure-driven reasons or to apply a variety of data generation measures ranging from the use of existing human and animal data, read-across and Quantitative Structure-Activity Relationship Modelling to alternative testing, i.e. in vivo testing. Intelligent testing strategies are requested to make optimal usage of all data generation sources and measures available.

However, as shown in the REACH Implementation Project 3.3. availability and quality of waiving tools significantly differ between individual toxicological endpoints and substances and expert knowledge is essential to establish and justify a sound intelligent data generation mix. Some of the animal tests can be replaced by a single alternative method, as was for skin corrosion or phototoxicity. However, the replacement of *in vivo* testing for systemic, repeated-dose and chronic toxicity represents major challenges, since these animal tests can be replaced only with integrated testing strategies including the use of (quantitative) structure-activity (QSAR) relationships, read-across approach; exposure considerations and *in vitro* methods, in order to obtain the data required for hazard prediction and / or risk assessment. Perspectives and challenges, focussing on the application of alternative measures to predict the most important endpoints of human health toxicity will be discussed in this article.

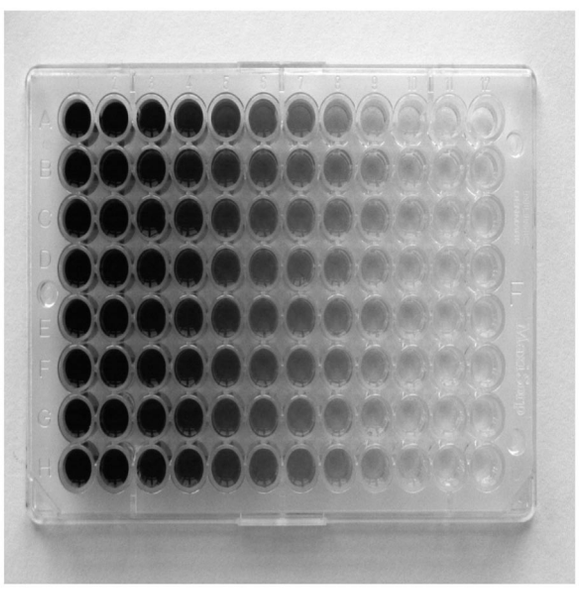
Acute systemic toxicity

Acute toxicity testing of chemicals is one of the first studies to be performed for safety management resulting in an estimation of the acute lethal dose (LD50). This test is a must for compounds in the tonnage band of 1 to 10 tons / year but may be obsolete if shown by other means that Phase-In-Substances are harmless. The procedure for testing acute oral toxic potential has been revised during the last years, moving to a reduced animal consumption. This process is continued with protocols for acute dermal and inhalative toxicity.

Under REACH there may be a regulatory requirement for the oral and dermal path of exposure. Drawing upon general principles of toxicity a study for dermal toxicity should only be performed if oral

toxicity has been detected and further requirements of REACH as given in Annex VIII are fulfilled. and dermal toxicity is expected to follow different modes of action. This is based on a data evaluation, which demonstrates that dermal toxicity do only occur when significant acute oral toxicity and relevant acute dermal irritation potential is observed (Gerner et al, 1994).

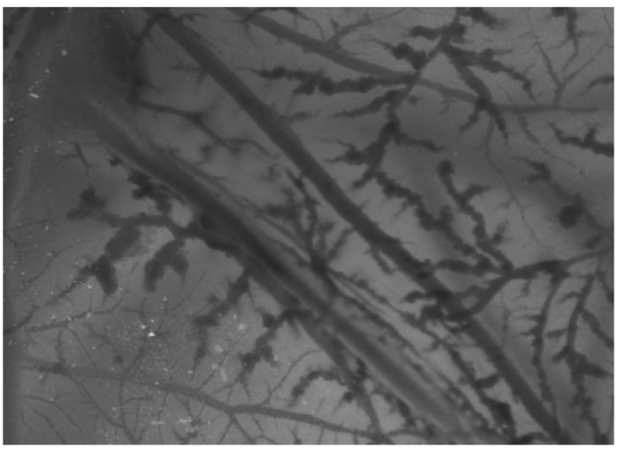
Since the early 1990's possibilities were evaluated to use in vitro tests for basal cytotoxicity in order to screen for systemic toxicity potentials and to predict lethal dose ranges and appropriate starting doses for acute systemic toxicity studies (NIH/ICCVAM 2001). This activity combined with development of appropriate QSAR methods and the use of existing but unpublished animal in-house data, can result in a replacement of animal testing for classification of acute toxicity in the near future.



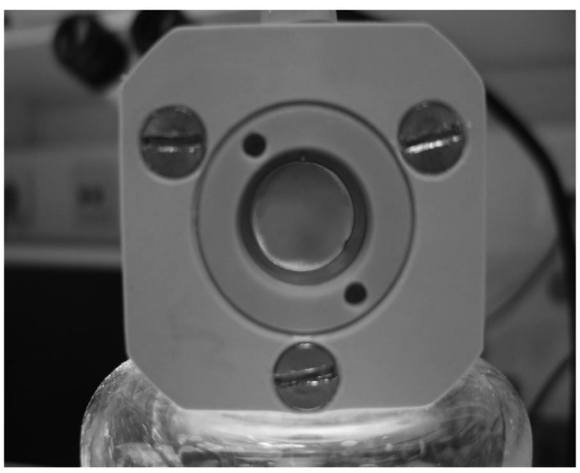
Photograph 1: Cytotoxicity assay – 96 well plate after treatment with a cytotoxic compound

Irritation and corrosivity

Irritation or destruction of skin or eyes is a well-known hazard of chemicals and therefore, as early in the 1940s rabbit tests had been developed by Draize and co-workers (1944). Several alternative in vitro testing systems for assessment of corrosion were developed, validated, and integrated into official guidelines (OECD TG 430 and 431). These should be applied to examine compounds within tonnages between 1 and 10 tons per year. At present acute local irriation has to be tested still on animals, since there is no validated in vitro method for classifying moderately skin-irritating chemicals. However, a validation study by ECVAM has been finished in 2006 including the human skin equivalent models, Episkin and EpiDerm. Promising results were obtained in this validation study where, 60 coded compounds in different laboratories were tested. Accroding to Annex VII of REACH such in-vitro-tests are required for substances between 1 and 10 tons year.



Photograph 2: HET-CAM Assay – Coagulation of blood vessels after treatment with an irritating compound



Photograph 3: BCOP assay – Turbidity of bovine cornea after treatment with an irritating compound

The existing OECD testing strategy excludes Draize tests on skin and eye only in case of severe lesions detected by in vitro systems or extrapolated from pH-data. However, testing for serious eye damaging is already routinely performed using alternative methods, although not formally validated. But it is expected that tests such as the BCOP test will meet the requirements of Annex VII.

Studies have identified physicochemical properties as being essential for causing significant local irritation. Also QSAR may become a useful tool for the prediction of the absence of relevant local irritation. It can be assumed that a significant reduction in in vivo testing for local irritation or corrosion will be necessary under future European chemicals regulation by using a tiered assessment strategy.

Sensitisation

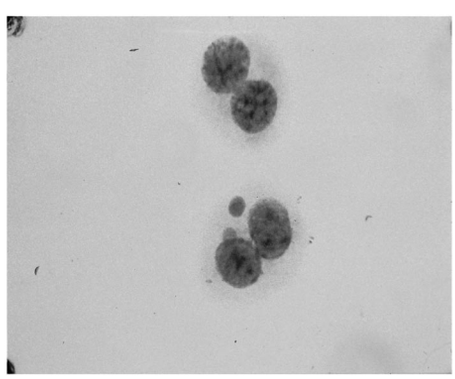
The allergenic potential of chemicals has become an important aspect in public health, and sensitisation has thus become an important part of the hazard and risk assessment process. In the past, approximately 90% of the necessary experiments were carried out according to the Magnusson-Kligman procedure. The Local Lymph Node Assay (LLNA) was introduced in OECD TG 429, a method using a smaller number of rodents and causing less pain. At present, no in vitro test for skin sensitisation has been validated but several systems are in the course of development, based on an improved understanding of the biochemical and immunological mechanisms underlying the process. REACH requires sensitisation testing under Annex VII, i.e. for small tonnages. In near future, skin sensitisation potential may probably be assessed by means of QASR predictions and the use of an in vitro tiered approach, including skin penetration, protein binding and reactivity to Langerhans cells.

Repeated dose toxicity

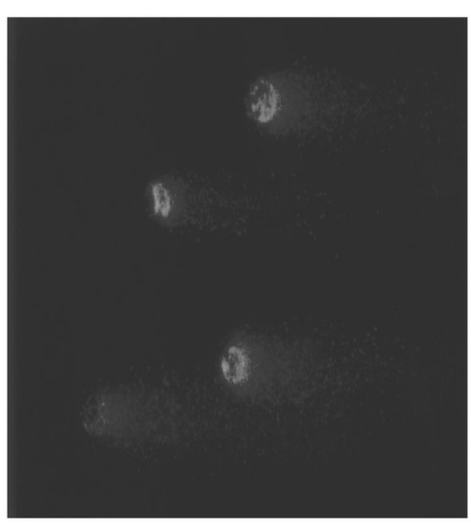
The identification of adverse health effects after repeated or long-term exposure to chemicals is based either on experimental animal studies or reliable human experience. Under REACH, a 28-day study will be required for chemicals equal to or above a production volume of 10 tons per year. The goal of a repeated dose toxicity study is the identification of potential target organs and the derivation of the No-Observed-Adverse-Effect-Level (NOAEL) or benchmark doses, which permit extrapolation necessary for risk assessment in humans. At present, no available alternatives to animal testing are acceptable for regulatory purposes aiming at detecting toxicity after repeated exposure. The major limitations to the use of in vitro models for the assessment of toxicity after repeated dosing are the lack of suitable in vitro systems to mimic all the possible interactions which may result in vivo, the limited possibilities of using cell culture systems to account for kinetics and biotransformation, and the difficulty to derive values such as NOAELs from in vitro systems. Experts in an ECVAM workshop held in 2006 agreed to focus on reducing the number of animals used for the purpose of such kind of studies. Integrated systems to predict NOAELs were discussed, using in vitro systems for determination of the No-Effect-Concentration (NOEC) in cells and tissues from different organs, the use of mathematical models describing the biokinetic processes (e.g. physiologically-based biokinetic modelling – PBBK) making use of a description of the physiological processes involved in the kinetics of chemicals in the body in relation to compound-specific parameters (such as partition coefficients between blood and tissues, protein binding, and metabolism). Also the use of biomarkers and the omics technologies may have to be included in the assessment process.

Genotoxicity

Genotoxicity testing is an important part of the hazard assessment of chemicals for regulatory purposes. The standard regulatory approach to genotoxicity testing is to use a tier-testing strategy with at least two in vitro tests in the first level. The in vitro tests have shown to be able to identify potential in vivo mutagens, but not all mutagens detected in vitro are relevant for the human situation. Therefore, positive results are often questioned by animal tests. In general, routine in vivo tests are conducted with somatic cells (mainly bone marrow). It may well be that available toxicokinetic and toxicodynamic data may give sufficient evidence to show whether the substance might pose a genetic hazard or not. It is of special importance whether a routine bone marrow test is appropriate (only in case of systemic availability) or a local genotoxicity test on highly exposed tissues will give more relevant information.

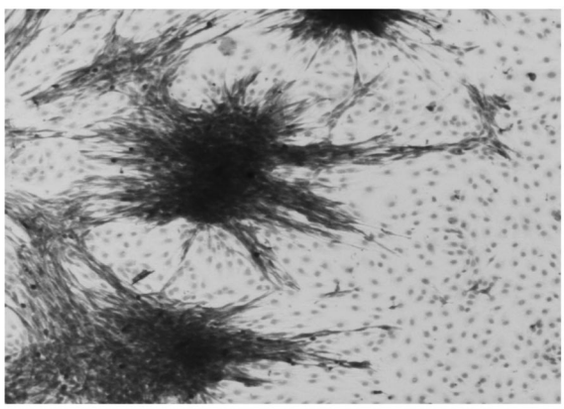


Photograph 4: Micronucleus assay in V79 cells – Formation of micronuclei after treatment with aneugenic compound



Photograph 5: COMET Assay in V79 cells after treatment of a genotoxic compound

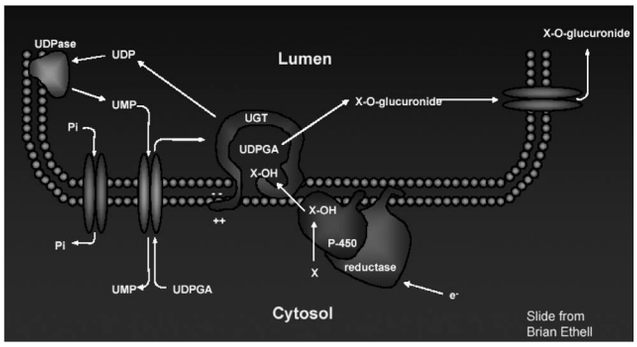
Under REACH the Ames-Test is required for tonnages exceeding one ton per year and may be followed by another mutagenicity test such as the HPRT if indicated. QSAR, expert systems, and in vitro approaches can be used immediately to prioritise chemicals for further testing on the basis of their potential genotoxicity. On this basis the number of animal experiments needed under the new chemical policy will stay low.



Photograph 6: Cell Transformation assay in Bhas42 cells after treatment with a carcinogenic compound

Carcinogenicity

The conventional approach to carcinogenicity testing is the life-time rodent bioassay in rats and mice of both sexes with full pathological analysis of all tissues. This assay is detecting complete carcinogens, as well as tumour promoters and co-carcinogens. Moreover, rat and mouse data do not correlate well, and extrapolating the information to humans is problematical. Data indicate that genotoxic chemicals are likely to exhibit trans-species carcinogenicity often in both sexes, whereas chemicals lacking any genotoxicity are exhibiting carcinogenicity, mostly manifested in one species, one sex and even in one specific tissue and usually at high dose levels. The non-genotoxic carcinogens cannot be predicted by the standard battery of gentotoxicity tests. At present there are a few in vitro methods (cell transformation systems) for detecting non-genotoxic carcinogens, but non of them has been validated for regulatory usage. Two cell transformation systems, the Balb/c3T3 and Syrian hamster embryo (SHE) cell assays are currently in the process of pre-validation. Modified protocols have improved the reliability and predictivity of these assays. Under REACH, carcinogenicity testing will follow a tiered approach considering all other toxicity and exposure data. REACH may require testing only if a tonnage of 1,000 tons / year is exceeded but points out that a carcinogenicity study may only be performed when there is evidence on a widespread use or long-term exposure to humans and there is a specific concern by a positive mutagenic potential or evidence on



Photograph 7: Diagram Metabolism

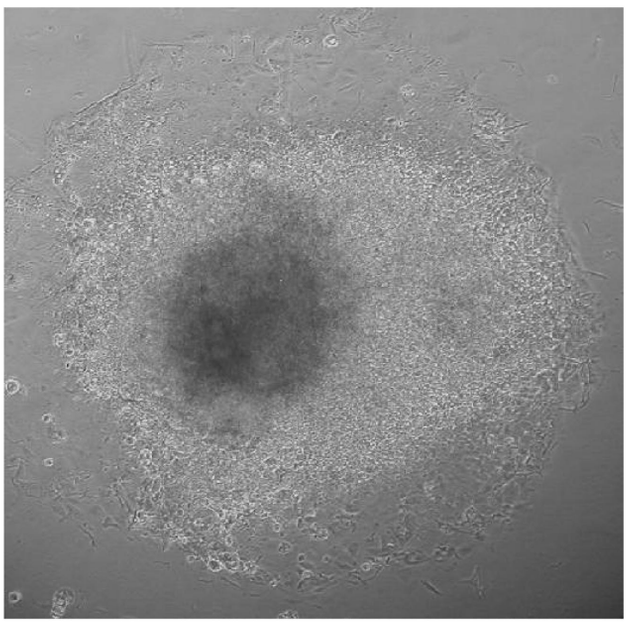
precursor lesions to neoplasm. This will limit the need for animal tesing.

Reproductive toxicity

REACH requires routine testing of reproductive toxicity if the compound exceeds a tonnage of 10 tons / year. Reproduction is a continuous cycle in which toxicity testing focuses on two key phases: on the period when male and female fertility could be impaired and on pregnancy in females, when developmental toxicity including teratogenicity could be induced. Several standardised OECD test guidelines are available for regulatory purpose. These tests require a huge number of animals. Thus more specific methods for detecting and studying endocrine effects are needed. Due to the complexity of the of the mammalian reproductive cycle, it is not possible to model the whole cycle in one or two in vitro systems in order to detect chemical effects on mammalian reproduction. But the cycle can be broken down to its biological components which can be studied individually or in combination. For the assessment of embryotoxic potential, the Mouse Embryonic Stem Cell Test (MEST) can be used. This test, together with two other in vitro methods (whole embryo culture and micromass test), were validated by ECVAM in the field of developmental toxicity. After this validation exercise the MEST was considered to be scientifically valid for distinguishing between non, weak/moderate, and strong embryotoxins. Taking the progress described into account, ECVAM took the lead to manage a conceptual framework in the area of reproductive toxicity (ReProTect) within the 6th Frame Work Program of the European Commission.

Evidenced based toxicology

Like in clinical medicine, toxicology has the opportunity to reshape and enlarge its methodology and approaches on the basis of compounded scientific knowledge (Hoffmann and Hartung, 2006). Such revision would have to be based on structured reviews of current practice, e.g. assessment of test performance characteristics, mechanistic understanding, extended quality assurance, formal validation and the use of integrated testing strategies.



Photograph 8: Differentiated mouse stem cell – Differentiation into a cardiomyocyte

All this can optimize the balance between safety, costs and animal welfare. Such an approach promises to make better use of resources and to increase the quality of results, facilitating their interpretation.

In the frame of REACH, the best way forward will be to develop integrated/intelligent testing strategies for assessing the hazard and risk linked to regulatory endpoints, integrating elements including in vitro tests, chemical categories (including read-across), in silico approaches and exposure considerations. At the same time, data generation strategies should not only be intelligent in the sense of sound science, i.e. applying all waiving tools available and deemed feasible. Data generation should also be efficient from a practical point of view, i.e. using all options that are legally foreseen and in compliance with common moral and ethic values in order to waste as few resources as possible on filling data gaps. This for instance may mean not to spend too much time on literature search for a melting point but focus efforts on saving animal lives by making all efforts to avoid a potentially unnecessary reproduction toxicity study. RCC is realizing the challenges in toxicology, is proactively including integrated testing strategies in the safety evaluation of chemical compounds and is supporting its clients with scientific and economic knowledge in the field of alternative testing.

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