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EURL ECVAM progress report on the development, validation and regulatory acceptance of alternative methods (2010-2013)

Prepared in the framework of Directive 76/768/EEC and Regulation (EC) No 1223/2009 on cosmetic products

Valérie Zuang, Michael Schäffer, Anita M. Tuomainen, Patric Amcoff, Camilla Bernasconi, Susanne Bremer, Silvia Casati, Paolo Castello, Sandra Coecke, Raffaella Corvi, Claudius Griesinger, Annett Janusch Roi, George Kirmizidis, Pilar Prieto, Andrew Worth, Sharon Munn, Elisabet Berggren, Maurice Whelan

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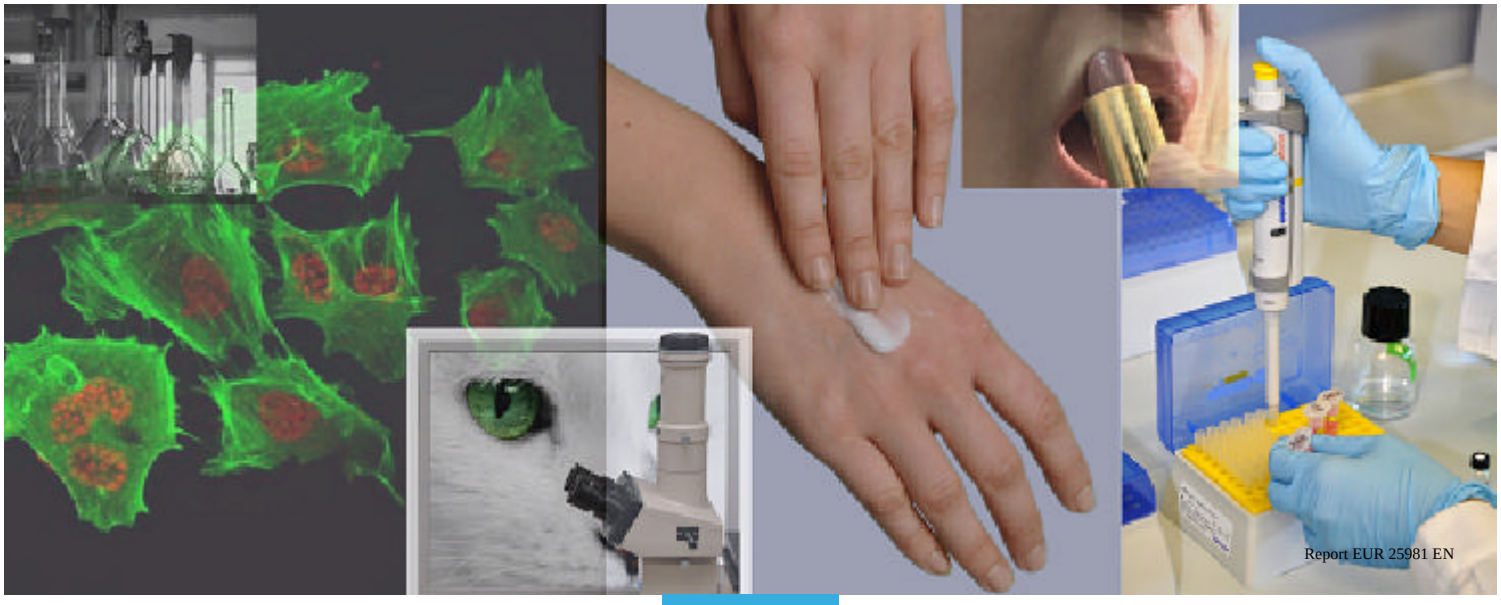

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Abstract

Provisions of Regulation No 1223/2009 on cosmetic products require that the European Commission reports on a yearly basis to the European Parliament and Council on the progress made in the development, validation and regulatory acceptance of alternative methods and on the compliance with the deadlines of the animal testing and marketing bans. This EURL ECVAM technical report provides an update since 2010 on the state of play of alternative methods for all the toxicological areas relevant to the Cosmetics Regulation and supplements the 2013 Commission Communication on the animal testing and marketing ban and on the state of play in relation to alternative methods in the field of cosmetics. Overall good progress has been made in the validation and regulatory acceptance in areas such as local toxicity where the underpinning science is more advanced and mature alternative methods are available. For very complex endpoints on the other hand, such as chronic systemic toxicity, carcinogenicity or reproductive toxicity, efforts are predominantly focused on research and development where the emphasis is on the integration of a variety of methods based on mechanistic understanding. The future is bright however, since considerable advances in new in vitro technologies, systems biology, bioinformatics and computational modelling are driving a paradigm shift in toxicological testing and assessment where non-animal methods will ultimately become the tools of choice.

[1] Directive 76/768/EEC (the "Cosmetics Directive") will be repealed and replaced by Regulation (EC) No 1223/2009 on cosmetic products as of 11 July 2013. The Regulation contains the same provisions on alternative methods as the Directive.

Table of Contents

Extended summary

Regulation No 1223/2009 on cosmetic products[2] requires that the European Commission reports on a yearly basis to the European Parliament and Council on the progress made in the development, validation and regulatory acceptance of alternative methods and on the compliance with the deadlines of the animal testing and marketing bans of animal-tested cosmetics stipulated therein. A detailed technical report describing the status of alternative methods for the toxicological areas falling under the 2013 marketing ban (*i.e.* repeated dose toxicity, toxicokinetics, reproductive toxicity, skin sensitisation and carcinogenicity) was prepared by the European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM) in collaboration with EU experts in 2011[3].

This EURL ECVAM technical report provides an update since 2010 on the status of alternative methods for all the toxicological areas relevant to the Cosmetics Regulation and supplements the 2013 Commission Communication on the animal testing and marketing ban and on the state of play in relation to alternative methods in the field of cosmetics[4]. A summary status update on the validation of alternative methods at EURL ECVAM and the regulatory acceptance of alternative methods is provided in Tables 1 and 2 of Annex 1, respectively. This information covers the period 2010 until today (1st quarter of 2013). For previous updates one should refer to the ECVAM technical report 2008-2009 (Zuang *et al.*, 2010[5]).

For acute local effects, such as phototoxicity and skin irritation and corrosion, where validated and adopted test methods already exist, continuing efforts have focused primarily on the optimisation and refinement of accepted test methods or on updating the existing Organisation for Economic Cooperation and Development (OECD) test guidelines so that their impact on the 3Rs (*i.e.* replacement, reduction and refinement of animal use) could be further increased and the test methods more widely used. This included in the area of phototoxicity the organisation of a Joint ECVAM-EFPIA Workshop on Phototoxicity for reviewing technical issues on the 3T3 Neutral Red Uptake Phototoxicity test (NRU-PT) and reflecting on strategies for improving the usability of the assay for non-topical pharmaceuticals. The workshop led to important recommendations which will be followed up by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

In the area of skin corrosion and in the framework of the OECD expert group on skin irritation/corrosion, OECD TG 431 was updated by further evaluating the currently available Reconstructed human Epidermis (RhE) test methods for skin corrosion testing in view of their ability to subcategorise according to UN GHS/EU CLP. In the area of skin irritation, OECD TG 439 was updated. Besides including now the Japanese similar test method

[2] EC (2009). REGULATION (EC) No 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *Official Journal of the European Union* L342/59 of 22.12.2009.
The Cosmetics Regulation repeals and replaces Directive 76/768/EEC (the "Cosmetics Directive") as of 11 July 2013 and contains the same provisions on alternative methods as the latter.

[3] Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J., Zuang V, *et al.* (2011). Alternative (non-animal) methods for cosmetics testing: current status and future prospects. Arch Toxicol. 85, 367-485.

[4] EC (2013). Communication from the Commission to the European Parliament and the Council on the animal testing and marketing ban and on the state of play in relation to alternative methods in the field of cosmetics. Brussels, 11.3.2013, COM(2013) 135 final.

[5] Zuang, V. *et al.* (2010) ECVAM Technical Report on the Status of Alternative Methods for Cosmetics Testing. JRC Scientific and Technical Reports. ISBN 978-92-79-16021-9. Available at: http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/animal_testing/at_ecvam_2008-2009_en.pdf. Accessed on 21.01.2013.

LabCyte EPI-MODEL, the test guideline also gained in clarity with regard to the main commonalities and differences of the various test methods' protocols included in the test guideline and of the underlying validation evidence for each of these methods. Numerous similar ("me-too") test methods were submitted to ECVAM for validation and peer review since the adoption of OECD TG 439 and the related Performance Standards for *In Vitro* Skin Irritation Test Methods based on Reconstructed Human Epidermis (RhE) in 2010.

In the area of eye irritation, several activities took place at various levels. At the regulatory acceptance level, the test guideline on the Fluorescein Leakage test method was adopted as OECD TG 460 in 2012. An OECD TG on the Cytosensor Microphysiometer was drafted and Performance Standards for the assessment of proposed similar or modified Cytosensor Microphysiometer test methods for eye irritation were annexed to the draft test guideline. In addition, OECD TG 437 on the Bovine Corneal Opacity and Permeability (BCOP) assay and OECD TG 438 on the Isolated Chicken Eye (ICE) test method were updated to allow the use of these two test methods also in a bottom up approach for the identification of chemicals not requiring classification.

At the validation level, the EURL ECVAM/Cosmetics Europe eye irritation validation study on two test methods based on reconstructed human tissue models, namely SkinEthic™ Human Reconstructed Corneal Epithelium (HCE) and EpiOcular™ Eye Irritation Test (EIT), arrived at a crucial decision point. The experimental data and the analyses on both test methods became available in 2012. While the reproducibility of both test methods was very high, some issues on predictive capacity for the SkinEthic™ HCE protocols and the EpiOcular™ EIT protocol for solids were encountered. However, analysis of the data of both test methods identified that the protocols could be improved. The optimisation of the EpiOcular™ EIT protocol for solids has been finalised and is currently assessed prior to conducting the remaining validation activity for this test method scheduled in 2013. On the other hand, the SkinEthic™ HCE protocol optimisation requires more time and will take place outside the eye irritation validation study.

To reduce or replace the Draize eye irritation test, testing schemes combining strengths of particular *in vitro* assays were proposed during a 2005 EURL ECVAM expert meeting. Based on the physicochemical properties and other existing data on the test material, the testing scheme proposes a "Bottom-Up" approach beginning with test methods that accurately identify non-irritants, or a "Top-Down" approach beginning with test methods that accurately identify severe irritants before progression of further *in vitro* testing. In the framework of a collaboration agreement between EURL ECVAM and Cosmetics Europe, analyses of *in vivo* Draize eye irritation data from various databases were carried out with the aim to use existing data to support the evaluation of *in vitro* methods and as such facilitate the development of testing strategies. In addition, analyses describing potential ways for combining *in vitro* tests methods in integrated testing strategies based on the concept of the Bottom-up and Top-down approaches were undertaken. Manuscripts describing the results of these studies are currently being prepared.

In the field of acute systemic toxicity, the EURL ECVAM validation study undertaken to evaluate the capacity of the 3T3 NRU cytotoxicity assay to specifically identify non-classified chemicals on the basis of the 2000 mg/kg body weight threshold introduced by the EU CLP Regulation was completed in 2011. The report was peer reviewed by the ECVAM Scientific Advisory Committee (ESAC) and the EURL ECVAM recommendation was issued in April 2013. The study confirmed that the test method has a high sensitivity and thus a low false negative rate (less than 5%), which means that substances found to be

negative (non-classified) in this test would most likely not require classification for acute oral toxicity under the CLP Regulation.

Similar findings were obtained in the EU FP6 ACuteTox project (2005-2010) in which cytotoxicity assays, organ-specific toxicity assays and biokinetic/metabolism methods were evaluated and combined in testing strategies for predicting human acute oral toxicity. The results of the ACuteTox project supported the use of the 3T3 NRU cytotoxicity assay within a tiered testing strategy to identify non-classified substances (oral LD_{50} > 2000 mg/kg body weight). The results also showed that complementing the 3T3 NRU assay with additional target organ specific *in vitro* assays provided added value by alerting for specific toxicity such as neurotoxicity, further reducing the number of under-predictions. Moreover, it was recognised that the inclusion of kinetic parameters in such strategies needs further evaluation. Other important European activities in this area focused on the identification of opportunities to avoid redundant testing. For instance, the value of acute toxicity testing by more than one route has been questioned and retrospective analyses of acute toxicity oral and dermal data revealed that testing for acute dermal toxicity is redundant for substances not classified for acute oral toxicity. An EPAA technical expert group is currently following up on those findings at regulatory level.

In the area of genotoxicity, work continued to focus on possible ways to improve the current *in vitro* testing strategy which generates a high false positive rate that leads to follow-up *in vivo* confirmatory tests that prove negative. Building on the progress triggered by the recommendations of the 2006 EURL ECVAM workshop, EURL ECVAM organised an expert meeting in January 2013 to tackle the question of how *in vitro* mammalian cell genotoxicity tests could reduce the need for *in vivo* follow-up testing with compounds positive in the Ames test. The findings of the meeting proved most promising and EURL ECVAM will now explore this avenue further. At OECD level, all test guidelines related to genotoxicity testing are currently being revised taking into consideration the recommendations defined in the EURL ECVAM workshop on false positives and follow-up work. The aim is to increase the quality of the *in vitro* data generated in accordance with the TG and consequently to avoid in some cases the need for follow-up confirmation of the results.

EURL ECVAM recently outlined a strategy for replacing animal testing for the purpose of skin sensitisation hazard identification and classification. Achieving this goal would satisfy the information requirements of several pieces of EU legislation and would thus have a large impact on the 3Rs. In addition it would contribute to a global approach for skin sensitisation assessment. The area of skin sensitisation is also one of the very few areas benefiting from the availability of a so-called Adverse Outcome Pathway (AOP) that describes the key mechanistic events that occur during the development of skin allergy, thus providing a theoretical basis for the design of integrated testing and assessment strategies. The test methods currently under development and validation by EURL ECVAM address key events of this skin sensitisation pathway.

Approximately ten test methods were submitted to EURL ECVAM between 2011 and 2012, most of which had been developed in the framework of the EU FP6 project Sens-it-iv on novel testing strategies for *in vitro* assessment of allergens that ended in 2010. Test submissions included proposals on the use of these methods in combination for hazard identification or potency predictions. Two test methods (DPRA and h-CLAT) were validated by EURL ECVAM and two test methods (DPRA and KeratinoSens that underwent an external validation study) were already also positively peer reviewed by the ESAC, while

the h-CLAT is still pending peer review. EURL ECVAM recommendations on the DPRA and KeratinoSens are currently being prepared. In parallel, EURL ECVAM has initiated an in-house project on the development of integrated testing strategies (ITS) (based on combinations of *in silico*, *in chemico* and *in vitro* methods) for chemicals hazard identification and classification. Since no regulatory accepted non-animal approaches for skin sensitisation yet exist, EURL ECVAM has submitted proposals to the OECD for the development of test guidelines on the test methods which underwent validation. The DPRA and KeratinoSens proposals were included in the 2012 OECD work program and the h-CLAT proposal was approved in April 2013.

In the Commission review of 2010, toxicokinetics was identified as an indispensable element for future non-animal testing approaches because it is needed to determine the internal dose that reaches target tissues and cells, at a given external exposure. This information is considered to be very important for the translation of *in vitro* studies to the human *in vivo* situation. In cosmetics safety testing, exposure assessment is the important first step to decide on the necessity for further testing. Only in cases where a cosmetic ingredient is bioavailable following dermal, oral or inhalation exposure further tests on systemic toxicity are necessary.

At the research and development level, activities being undertaken by EURL ECVAM include for example the integration of toxicokinetic modelling into the prediction of *in vivo* dose-response curves without animal experiments, the development of a modelling approach to predict human bioaccumulation, design of a three dimensional metabolic test system for both toxicokinetic and toxicodynamic applications, and the combination of *in vitro* and *in silico* methods to predict target organ effects on humans under repeated dose exposure.

The cytochrome P450 (CYP) induction validation study using the human cryopreserved HepaRG® cell line and cryopreserved human hepatocytes is foreseen to end in spring 2013 and the ESAC peer review to start in autumn 2013. Considering the good experimental results of the CYP-induction validation study, a Standard Project Submission Form (SPSF) for the development of a Performance-based Test Guideline (PBTG) on the establishment of human-derived hepatic system to investigate biotransformation and toxicity of compounds, by evaluation of cytochrome P450 induction competence, was submitted to OECD and recently accepted onto its Test Guidelines work programme.

In the area of carcinogenicity, an OECD TG on the Cell Transformation Assay (CTA) in Syrian Hamster Embryonic (SHE) cells is currently under finalisation and has been submitted to the OECD WNT[6]. The TG is based on the EURL ECVAM study designed to address standardisation, transferability and reproducibility of three Cell Transformation Assays protocol variants for the SHE CTA (at pH 6.7 and pH 7.0) and the BALB/c 3T3 assay. CTAs are considered to provide additional useful information to the routinely employed tests for assessing carcinogenic potential and are therefore included in various recent guidelines and testing strategies for such purposes.

A full replacement of regulatory animal testing in the area of carcinogenicity requires however strategies to assess both genotoxic and non-genotoxic carcinogens. EURL ECVAM continues to pursue its strategy to improve testing for genotoxicity as described above. However, for non-genotoxic carcinogenicity assessment no strategy is in place yet in the scientific and regulatory community due to multiple unknown mechanisms of action and

[6] Working Group of National Coordinators of the OECD Test Guideline Programme

lack of sufficient knowledge of the cellular and molecular events underlying carcinogenicity.

In the area of reproductive toxicity, some *in vitro* tests have been developed and validated that address specific aspects such as embryotoxicity or endocrine disrupting effects. This reflects the fact that for the moment only selected mechanisms which may lead to reproductive toxicity can be simulated *in vitro*. These *in vitro* tests are typically used as screening tools or for providing supporting mechanistic information when carrying out safety assessments, but have limited impact from a 3Rs perspective.

As with other complex health effects or endpoints, the main problem in the area of reproductive toxicity is the lack of understanding of the many mechanisms of action leading to toxicity. In that sense, the current efforts to establish databases aiming at the identification of the most sensitive targets and associated mechanisms of reproductive toxicants is a promising way ahead. Efforts are also currently invested in the optimisation of the validated embryonic stem cell test in particular for improving its predictions and this is reflected in the tests recently submitted for validation.

The need to screen chemicals for endocrine activity and other regulatory requirements related to endocrine disrupting chemicals have triggered the development of *in vitro* assays designed to detect a range of different activities, such as the potential of a chemical to interact with hormone receptors. In fact, several test methods measuring the capacity of chemicals to influence hormonal pathways, developed within the EU FP6 project Reprotect, were submitted to EURL ECVAM in 2010. These were either binding assays (*i.e.* androgen receptor binding assay) or transcriptional assays measuring either (anti-) androgenic activity or (anti-) estrogenic activity. Some of these tests are in the process of validation by EURL ECVAM. These assays, when validated, will contribute to OECD performance-based test guidelines for estrogen receptor and androgen receptor transactivation assays.

For repeated dose systemic toxicity testing, some *in vitro* and *in silico* methods have been developed which may be useful for obtaining mechanistic information or in some cases for identifying the potential hazard of substances and their potential effect on certain target organs. However the methods available still fall very short of providing all the information needed for quantitative safety assessment.

To tackle the challenge of assessing chemicals for repeated dose systemic toxicity using alternative methods, a major research initiative is underway in the form of a EU FP7 project called SEURAT-1 ("Safety Evaluation Ultimately Replacing Animal Testing"). Its research strategy is to adopt a toxicological mode of action framework to describe how a substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and *in vitro* models that predict quantitative points of departure needed for safety assessment. Similarly, but more focused on pharmaceuticals, the EU FP7 project Predict-IV ("Profiling the toxicity of new drugs: a non-animal-based approach integrating toxico-dynamics and biokinetics") aims to develop strategies to improve the assessment of drug safety in the early stage of development and late discovery phase by an intelligent combination of non-animal test systems, cell biology, mechanistic toxicology and *in silico* modelling. A strategy for measuring the real exposure of cells to the drug and/or their metabolites has been developed. By combining then the biological effects with toxicokinetics and modelling, the project aims to link exposure and effect.

In line with the European Union activities to promote alternatives to animal experimentation and the commitment of the Joint Research Centre to enhance the dissemination of information on advanced and alternative methods, EURL ECVAM is managing a DataBase service on ALternative Methods to animal experimentation (DB-ALM). The key feature of the DB-ALM is to provide user-oriented documentation in the form of quality controlled descriptions of available alternative approaches in the different fields of science which are prepared by experts and are ready for immediate use. ECVAM's information systems have recently been complemented by the EURL ECVAM Search Guide that has specifically been developed to inform and support untrained database users to find high quality information on relevant alternative methods and strategies in an easy, systematic and efficient way.

The 2010 Commission review on the status of alternative methods addressed in particular the five toxicological areas falling under the 2013 marketing ban (*i.e.* repeated dose toxicity, skin sensitisation, carcinogenicity, toxicokinetics and reproductive toxicity). It concluded that full replacement of animal testing for skin sensitisation may be the next achievable milestone in 3Rs in the coming years. Comprehensive alternative approaches to toxicokinetic assessment of chemicals were not foreseen for at least five to seven years, with complete replacement of animal testing coming some years after. For repeated dose toxicity, carcinogenicity and reproductive toxicity, the time horizon for full replacement was not speculated since the review concluded that a considerable amount of research was still needed. Although the general conclusions drawn in the 2010 Commission review still seem valid, the considerable progress made since then on many fronts indicates that sustained effort and investment in understanding toxicology and developing innovative methods will undoubtedly reduce the current reliance on animal testing.

1. Introduction

A complete marketing ban on animal-tested cosmetics entered into force in March 2013 following Directive 76/768/EEC (EC, 1976) and Directive 2003/15/EC (EC, 2003). One provision of the Cosmetics Directive is that the European Commission reports regularly to the European Parliament and Council on the progress made in the development, validation and regulatory acceptance of alternative methods and on the compliance with the bans. In 2011, it was furthermore asked to raise any potential technical difficulties in complying with the 2013 marketing ban. In that context, during 2010 the Commission reviewed the status of development of alternative methods and future prospects in five toxicological areas, namely repeated dose toxicity, skin sensitisation, carcinogenicity, toxicokinetics and reproductive toxicity. The EURL ECVAM (European Union Reference Laboratory for Alternatives to Animal Testing) of the European Commission's Joint Research Centre chaired the five established working groups composed of nominated EU experts and coordinated the drafting of the resulting report (Adler *et al.*, 2011). The main outcome of this extensive review was that timelines could only be estimated for the full replacement of animal tests in the area of skin sensitisation (*i.e.* 2017-2019) which included the possibility to generate potency information. Alternative methods able to simply discriminate between skin sensitisers and non-sensitisers were estimated to become available earlier. No specific timeline could be estimated in the areas of toxicokinetics, repeated dose toxicity, carcinogenicity and reproductive toxicity due to the many underlying scientific challenges in these areas. The forecasts for the full availability of alternative test methods made in the 2010 report did not diverge much from estimates provided in a similar review already conducted by the Commission in 2005 (Eskes and Zuang, 2005).

This EURL ECVAM technical report provides an update since 2010 on the status of alternative methods for *all* the toxicological areas relevant to the Cosmetics Directive and supplements the 2013 Commission Communication on the animal testing and marketing ban and on the state of play in relation to alternative methods in the field of cosmetics (EC, 2013).

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Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J., Zuang V, *et al.* (2011) Alternative (non-animal) methods for cosmetics testing: current status and future prospects. Arch Toxicol. 85, 367-485.

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Eskes and Zuang (2005). Alternative (Non-Animal) Methods for Cosmetics Testing: Current Status and Future Prospects. Alternatives to Laboratory Animals (ATLA), volume 33, supplement 1, July 2005.

2. Toxicological areas falling under the 2009 marketing ban

2.1 Acute Systemic Toxicity

2.1.1 Brief description of the toxicological area

The term acute systemic toxicity comprises the general adverse effects that occur after a single or multiple exposure of an animal to a substance within 24 hours and during an observation period of at least 14 days. The *in vivo* studies carried out to assess acute systemic oral, dermal and inhalation toxicities are described in the Organisation for Economic Cooperation and Development (OECD) test guidelines (TGs) for the testing of chemicals. For the oral route they include three refinement and reduction alternative methods described in TG 420 (fixed dose procedure), TG 423 (acute toxic class method) and TG 425 (up and down procedure) (OECD, 2001a, b, c). For acute dermal toxicity, the only guideline available is the classical dermal LD_{50} study (TG 402, OECD, 1987) and for inhalation toxicity there is a revised version of the classical LC_{50} study (OECD, 2009a) and the acute toxic class method (TG 436, OECD, 2009b), which allows to reduce considerably the number of animals used. The endpoint measured in these studies is animal morbidity or death with the exception of the fixed dose procedure that measures evident toxicity. One of the main purposes of conducting the test is to categorise substances according to their potential hazards and the dose required to cause toxicity (*i.e.* classification and labelling).

A major gap at present in this area is our lack of understanding of all *in vivo* mechanisms of action of chemicals. At the ICCVAM/ECVAM/JacVAM workshop on acute chemical safety testing, where approaches that could help to identify key toxicity pathways for acute oral toxicity were discussed, it was agreed that the mechanistic information could be used to develop more predictive *in vitro* test methods and to identify earlier and more humane endpoints during *in vivo* testing (NIH, 2009). Several international programmes have explored in the past the possibility to use cell-based methods to predict acute oral toxicity. Among them, the Multicenter Evaluation of *In Vitro* Cytotoxicity (MEIC) programme concluded that the majority of chemicals evaluated within the project appeared to be acutely toxic to humans by basal cytotoxicity, that is, by interfering with general cell functions common to all cells (Ekwall, 1999).

2.1.2 Development/optimisation/improvement of alternative methods

The EU FP6 ACuteTox project (2005-2010) aimed to develop a non-animal testing strategy for predicting human acute oral toxicity by evaluating and combining cytotoxicity assays, organ-specific toxicity assays (*i.e.* haemato-, neuro-, nephro- and hepatotoxicity) and biokinetic/metabolism methods (Kinsner-Ovaskainen *et al.*, 2012). More than 50 different *in vitro* and *in silico* methods have been evaluated and 124 chemicals tested (AXLR, 2010, 2011). The outcome of this research project with regard to the assessment of the predictive capacity of the proposed tiered testing strategies and the identification of assay combinations that give best prediction in terms of classifying the chemicals into the EU Classification, Labelling and Packaging (CLP) Regulation acute oral toxicity categories (EU, 2008), has reinforced other results obtained with the 3T3 NRU cytotoxicity assay (Prieto *et al.*, 2013), supporting its use within a tiered testing strategy to identify non-classified substances (oral LD_{50} > 2000 mg/kg body weight). The results also showed that complementing the 3T3 NRU assay with additional target organ specific *in vitro* assays provided added value by alerting for specific toxicity (*e.g.* neurotoxicity) and further reducing the number of under-predictions (Prieto *et al.*, 2012; Zurich *et al.*, 2012). In general, the ACuteTox strategies had a tendency to over-predict toxicity, which was explained by their failure to capture *in vivo*

biokinetics (*i.e.* restricted access to the target tissue, quick elimination or detoxification through metabolism). The importance of incorporating suitable kinetic parameters in testing strategies for acute oral toxicity is widely acknowledged (Adler *et al.*, 2011; Coecke *et al.*, 2012; Blaauboer *et al.*, 2012; Broeders *et al.*, 2013).

A range of Quantitative Structure-Activity Relationships (QSAR) models have been developed to predict acute systemic toxicity (LD_{50} values or classifications) via various routes, as reviewed by Lapenna *et al.*, (2010). Such models are generally statistically-based models, supported by little or no mechanistic rationale, but they may nevertheless be predictive for specific chemical classes. In a recent JRC study (Norlén *et al.*, 2012) four QSAR models and the 3T3 NRU *in vitro* prediction model were applied to a dataset of 180 chemicals. The results showed that the QSAR models have a predictive capacity (correlation with LD_{50} of 49-84%, depending on the model) equivalent to or greater than the 3T3 NRU *in vitro* test (correlation with LD_{50} of ~50%). The use of the five models in combination did not yield a significantly better predictive performance, but it did enable the classification of chemicals to be optimised for overall accuracy, sensitivity or specificity, according to the end-user's requirements. For instance, models can be combined differently to maximise sensitivity for identification of toxic chemicals or to maximise specificity for identification of non-toxic chemicals, at the expense of a higher false positive or false negative rate, respectively.

One of the efforts of the European Partnership for Alternative Approaches to Animal Testing (EPAA) in the area of acute systemic toxicity is to identify opportunities to avoid redundant testing (Seidle *et al.*, 2010; EPAA, 2010). The value of acute toxicity testing by more than one route has been evaluated in several publications (Indans *et al.*, 1998; Thomas and Dewhurst, 2007; Creton *et al.*, 2010; Seidle *et al.*, 2011; Andrew and Wright-Williams, 2011). These analyses have shown that testing for acute dermal toxicity is redundant for substances not classified for acute oral toxicity, which casts doubt on the need for acute dermal toxicity as a default information requirement in safety assessment and CLP. The data requirements under the recently adopted Biocidal Products Regulation (EU, 2012) state that testing via other routes is only necessary when specified criteria are met. A document containing the technical progress made in some areas has been recently prepared by an EPAA technical expert group with the ultimate goal to invite the European Commission to consider similar improvements in other EU legislation (*e.g.* REACH; EC, 2006).

2.1.3 Test Method Submissions

The Dequenching After Photobleaching assay (DAP) was received in 2011. This is a quantitative mechanistic-based assay that addresses DNA alteration in association (or not) with cell death. The technology used is based on fluorescence dequenching after photobleaching. The test method was not prioritised since its scientific and regulatory relevance and level of development were unclear.

2.1.4 Validation of alternative methods

As a follow-up to the NICEATM/ECVAM validation study on the prediction of acute oral toxicity by NRU cytotoxicity assays (NIH, 2006) and assuming that most industrial chemicals are not likely to be acutely toxic (Bulgheroni *et al.*, 2009), ECVAM has coordinated a second validation study to evaluate the capacity of the 3T3 NRU cytotoxicity assay to specifically identify non-classified chemicals on the basis of the 2000 mg/kg b.w. threshold introduced by the EU CLP Regulation. The results have shown that the test method has a high sensitivity (about 95%) and hence a low false negative rate (less than 5%), which means that substances found to be negative (non-classified) in this test would most likely not

require classification for acute oral toxicity under the CLP Regulation. Conversely, the low specificity of the assay (about 42%) means that the test overpredicts many negatives as positives (false positive rate of 58%), which means that the test cannot be used on its own for the identification of substances requiring classification (Prieto *et al.*, 2013). This validation study was completed in 2011 and the report was peer reviewed by the EURL ECVAM Scientific Advisory Committee (ESAC). The ESAC opinion served as basis for the EURL ECVAM recommendation available on the IHCP website (http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/eurl-ecvam-recommendations).

When the 2000 mg/kg threshold was applied to the data set from the NICEATM/ECVAM validation study (NIH, 2006) and from Schrage *et al.*, (2011), which cover a wide range of chemical uses, a similar high sensitivity and low negative rate was obtained. In the light of these results, it is questionable whether many acutely toxic chemicals really act via a specific mechanism without also showing any cytotoxicity.

Schrage *et al.*, (2011) discussed mainly the use of NRU cytotoxicity data for predicting the *in vivo* classification confirming the low overall concordance of 35% also found in the NICEATM/ECVAM validation study (NIH, 2006). It was also reported that the prediction of the starting dose for the subsequent *in vivo* test was useful with regard to potential reduction in animal usage for 59% of the 203 substances tested.

2.1.5 Regulatory acceptance of alternative methods

To date the cytotoxicity assays are recognised only as additional tests that can be used for estimating the initial doses for acute oral systemic toxicity tests *in vivo.* Based on the results of the NICEATM/ECVAM validation study, OECD has adopted a Guidance Document (GD No 129) that describes methods to determine the *in vitro* basal cytotoxicity of test substances using NRU assays and the use of the *in vitro* data to determine starting doses for *in vivo* acute oral systemic toxicity tests (OECD, 2010).

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2.2 Skin irritation/corrosion

2.2.1 Brief description of the toxicological area

Local, topical exposure to chemicals can lead to adverse skin effects. According to the severity and reversibility of effects one distinguishes **skin corrosion** from **skin irritation**. In the industrialised world, adverse skin effects occurring as a consequence of exposure to industrial chemicals are a major concern of occupational and consumer safety.

2.2.1.1 Skin corrosion

Mechanistically, corrosion is a very simple endpoint: corrosive substances damage the exposed skin area leading to necrosis (death) of the skin tissue beyond repair. As a consequence, the affected area can be regenerated only from the healthy skin surrounding the necrotic patch.

2.2.1.2 Skin irritation

In contrast irritant substances lead to a reversible local inflammatory reaction caused by the innate (non-specific) immune system of the affected tissue. Elements of a putative Adverse Outcome Pathway (AOP) for irritation can be described as follows: Inflammation is a physiological response of any tissue to injury and trauma. Chemically-induced skin inflammation is triggered by cell damage and tissue trauma. Skin cells release inflammatory mediators (chemokines and cytokines, *e.g.* interleukines 1 alpha or 8) which increase the diameter and permeability of blood vessels, attract immune cells (*e.g.* mast cells, neutrophils) to the site of injury and trigger the migration of immune cells through the endothelium (leukodiapedesis) into the tissue where they participate in antigen clearance and tissue repair. Moreover inflammatory mediators may stimulate nerve endings leading to symptoms such as burning, itching or stinging sensations. This local inflammatory response leads to the classical clinical symptoms of skin irritation: rubor (redness), warmth (calor), painful sensations (dolor) and also the swelling (oedema) of the tissue area affected. Redness and warmth of the skin tissue are consequences of increased blood flow at the inflamed area. The swelling of the skin is due to increased permeability of the endothelial cells forming the walls of blood vessels.

2.2.1.3 Acute versus cumulative irritation

While some chemicals will only trigger an irritant response after repeated exposure of the same skin area (cumulative irritants), other chemicals will even after a one-time exposure cause irritation (acute irritants). Although cumulative irritation clinically is more frequent than acute irritation, current regulatory requirements focus on the assessment of the acute irritation potential of chemicals in order to support the risk management associated with the production, handling and transport of chemicals, but also possible exposure to acutely irritant and corrosive substances contained in various products.

2.2.1.4 Legislations requiring data on possible adverse skin effects

Data on skin irritation/corrosion effects are required by several pieces of legislations, notably

- the **Classification, Labelling and Packaging (CLP) Regulation** (1272/2008; EC, 2008a)
- the **Cosmetics Directive** (76/768/EEC)**, which will be repealed, from July 2013 onwards, by the EU regulation on cosmetic products** (EC 1223/2009; EC, 2009)
- **the REACH Regulation** (1907/2006; EC, 2006)

Brief outline of Classification and Labelling for adverse skin effects
The EU CLP Regulation implements the UN GHS for classification and labelling (C&L). The C&L categories used are based on visually observable effects in rabbit skin following exposure (Draize skin corrosion and skin irritation test).

- ***Corrosive substances*** are labelled 'Category 1' in agreement. This category contains three further optional subcategories which correspond to the UN Packing Groups I, II and III for the transport of goods. The subcategories are implemented in the EU. They differ with regard to the exposure times required to elicit skin corrosion in the rabbit and are referred to as 1A ("strong corrosive"), 1B ("moderate corrosive") and 1C ("mild corrosives").

- ***Irritant substances*** are labelled 'Category 2'. While UN GHS allows furthermore the use of an additional "opt-in" category to classify mild irritants ('Category 3'), this optional category is not implemented in the EU and hence, C&L of skin irritants in the EU follows a dichotomous categorisation: Category 2 versus non-classified.

Table 3 (section 2.2.5) provides an overview of the available and accepted traditional and alternative test methods in the area of skin irritation and corrosion and flags new developments associated with some of these standardised testing methods.

2.2.2 Development/optimisation/improvement of alternative methods

Having validated reconstructed human epidermis (RhE) -based *in vitro* skin corrosion (Fentem *et al.*, 1998; Barratt *et al.*, 1998) and irritation test methods (Spielmann *et al.*, 2007) and having acted as lead organisation for the drafting and regulatory acceptance of TG 439 on *in vitro* skin irritation addressing the needs of the UN GHS / EU CLP system (Griesinger *et al.*, 2010), EURL ECVAM is continuing to follow the development of this area with respect to new technologies, assays and emerging concepts of production of reconstructed tissues (see also: Griesinger C, Zuang V, Section "Skin Irritation and Corrosion" in Bouvier d'Yvoire *et al.*, 2012).
The OECD expert group on skin corrosion and irritation brings together scientists from various OECD member countries, the Commission (ECVAM), regulators, test method developers as well as validation experts. Most activities towards the further improvement of test methods have taken place in the context of this expert group. The different activities are summarised below (points 2.2.2.1 to 2.2.2.4) and is complemented by reflections on new proposed production means (point 2.2.2.5):

2.2.2.1 Update of TG 439 on *in vitro* skin irritation testing (lead: ECVAM)

Following a performance-based validation study of the Japanese LabCyte EPI-MODEL (manufactured by J-Tec, Ltd), the Japanese Centre for the Validation of Alternative Methods had requested an evaluation of this study by the OECD expert group. Following positive evaluation of the (amended) study by the OECD expert group, the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) decided in 2012 to include this assay in TG 439. On request of the OECD Test Guidelines Programme, ECVAM acted as lead organisation and prepared, in collaboration with JaCVAM, the necessary update of TG 439, taking the opportunity to amend the test guideline also with regard to a) the clarity of presentation of the underlying validation evidence for each of the methods and b) an overview on the main commonalities and differences of the various test methods' protocols included in TG 439. The latter should support test users with regard to selecting the test methods that meets their specific requirements. Such a comparison table adds moreover to the transparency of the test methods included in the TG and may become a standard element

in Performance-Based Test Guidelines (PBTGs). The updated version is currently (January 2013) scheduled for discussion at the next WNT meeting in April 2013.

2.2.2.2 Update of TG431 on *in vitro* skin corrosion testing using RhE test methods

Currently the RhE-based test methods for skin corrosion testing are accepted to distinguish corrosive substances (Category 1) from substances considered non-corrosive or non-classified (*c.f.* TG431 and test method B40.bis). The UN GHS however foresees three further optional corrosive subcategories (1A, 1B, 1C) which are fully implemented in the EU through EU CLP. Notably, one of these RhE test methods, the EpiSkin, had originally been validated by ECVAM for subcategorisation of corrosives into R35 and R34 [previous Dangerous Substance Directive (DSD) system]. These categories correspond currently to subcategories 1A (= R35) and a combination of 1B and 1C (= R34). Information on subcategorisation is considered particularly important for the transport of chemicals: the three hazard subcategories directly correspond to UN packaging groups I to III. These differ substantially with regard to the restrictions imposed on the packaging and transport of substances (*e.g.* packaging size, dimension and quality of packaging material, means of transport *etc.*). Therefore, information on subcategorisation and hence packing groups would be useful additional information for chemicals producers and their downstream users in view of economising on packaging needs of chemicals that can be demonstrated to fall in packing groups II and III.

To address this need, the OECD expert group on skin corrosion and irritation agreed that, on the background of the successful original validation of the EpiSkin (Fentem *et al.*, 1998) for partial subcategorisation (R35 and R34), the currently available suite of RhE test methods for corrosion testing should be further evaluated in view of their ability to subcategorise. One of the major driving forces was the *availability of new control experiments*, not available during original validation. These control experiments address possible false predictions associated mainly with chemicals (a) that directly reduce the viability dye used in the protocols [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)] or (b) interfere with an accurate measurement of optical density of formazan, the MTT reduction product (*e.g.* colorants).

The projects provided the following results:

- None of the methods was able to resolve all three subcategories. However one test method was able to reliably and accurately predict category 1A versus a combination of categories 1B and 1C versus non-classified substances.
- The remaining test methods showed a high rate of over-predictions of 1B/1C categories as 1A (ca 45% over-prediction rate).
- While this is not a concern from a precautionary point of view, it is an unacceptably high rate of false positive predictions. ECVAM has therefore suggested considering 1A predictions from these test methods as unresolved Category 1 predictions. This has been taken up in the draft TG (adopted by WNT in 2013). Thus, these test methods are able to predict Category 1, a combination of categories 1B and 1C and non-classified substances.
- A very useful outcome of this project was the definition of a suitable list of reference chemicals (n=30) which have been included in the updated Performance Standards of TG 431 and also TG 430.

2.2.2.3 Update of TG 430 on *in vitro* skin corrosion testing using transcutaneous electrical resistance (TER) measurements

The update concerns the definition and inclusion of Performance Standards as Annex 1 to the TG. These PS will support the ready assessment and validation of similar and modified TER-

based test methods in accordance with the principles of Guidance Document No. 34. The list of reference chemicals suggested in the updated version of the TG is identical to the one suggested in TG 431.

2.2.2.4 Refinement of the testing strategy for skin corrosion and irritation defined during implementation of the REACH regulation and published in the relevant ECHA guidance

During the REACH implementation project an Integrated Testing Strategy (ITS) for skin corrosion and skin irritation had been developed within an endpoint working group chaired by ECVAM (see ECHA guidance, 2012). One of the sub-projects within the OECD expert group for skin corrosion and irritation aimed at refining this ITS. So far work has been limited to a more consistent and explicit description of the elements of the ITS, *i.e.* the test methods' predictive capacity, reliability, applicability and limitations. Further work aiming at the derivation of possible weighing factors attached to the various data sources is on-going under the lead of Germany. The conceptual approach chosen is to define a consistent data matrix of chemicals with credible reference data and analyse the relative contribution of each data source to the decision making using a semi-quantitative approach. The derived factors can then be attached as weighing factors to the individual test methods when conducting a weight-of-evidence analysis of chemicals.

2.2.2.5 Automated production of reconstructed human epidermis

A new development that deserves attention is the automation of the production of reconstructed human epidermis. As epidermal models are still rather costly and could, in addition for skin corrosion/irritation purposes possibly also be employed for other endpoints (*e.g.* genotoxicity, percutaneous absorption), economical ways of producing these tissues may support employing human based tissue models in a variety of toxicological assessments. An automated production facility of open source reconstructed human epidermis (OS-Rep) models has been installed by Fraunhofer Gesellschaft in Germany[7]. Important parameters such as batch quality and stability as well as robustness and relevance of this tissue model remain to be established and independently evaluated.

2.2.3 Test Method Submissions

Three test submissions of skin irritation test methods, all based on RhE-technology, were submitted to EURL ECVAM in the time period of 2009-2012. Two of these methods have already been evaluated by ECVAM with respect to their similarity to validated reference methods. Based on a comparison of the essential test method components and the protocol parameters with the ECVAM Performance Standards (EURL ECVAM, 2009), it was established that these two methods are sufficiently similar to qualify for PS-based validation. One other method that has been pre-submitted to ECVAM in 2009 is still in the development phase. From a tissue engineering perspective all three test methods use simple reconstructed epidermis as test system.

Notably, one of the test methods, the OS-Rep, is based on an "open source" concept. This test method was originally developed by Poumay and co-workers and has been published without any restrictions (Poumay *et al.*, 2004). The "open source" concept would carry this further: all necessary information on both the production and maintenance of OS-Rep (*i.e.* the relevant standard operating procedures) will not be subject to any restrictions (*e.g.* patents or other intellectual property rights) as is the case with the current tissue models marketed by the various producers. The idea is that all interested users (either in academia, in

[7] Link to the relevant page at Fraunhofer Gesellschaft:
http://www.igb.fraunhofer.de/en/competences/tissue-engineering/tissue-models/skin-from-the-factory.html

research or contract-research organisations) will be able to reconstruct the RhE test system in their facilities and use the test system either in association with the relevant protocol for skin irritation testing (*i.e.* as skin irritation test method) or for other purposes (*e.g.* research and development).
This approach is novel to an area of complex tissue-engineered test systems which, so far, are all manufactured and quality-controlled by the original test method producers who sell batch-controlled tissue kits with elements that are protected by intellectual property rights.
EURL ECVAM is monitoring this development. In particular the issues of (1) *transferability* of the reconstructed model and the implementation of appropriate (2) *batch quality control procedures* for tissue models reconstructed in individual user laboratories need to be carefully evaluated.

2.2.4 Validation of alternative methods

There are currently no test methods for *in vitro* skin irritation testing under validation at EURL ECVAM. However, EURL ECVAM continues to receive submissions of external test method validation studies performed in reference to the EURL ECVAM Performance Standards (EURL ECVAM, 2009). These submissions are evaluated by EURL ECVAM in view of establishing their compliance with the Performance Standards-based requirements. In case of compliance EURL ECVAM advises the test method developer to conduct an external Performance Standards-based study or, if such a study has already been submitted to EURL ECVAM, forwards these to ESAC for independent scientific peer review. Currently, two of the three test methods submitted between 2009 and 2012 are under external validation.

2.2.5 Regulatory acceptance of alternative methods

Currently, internationally accepted test methods for skin irritation testing include the traditional animal test (Draize rabbit test) as well as *in vitro* test methods. Currently, all accepted *in vitro* test methods for skin irritation are based on the RhE-technology validated by EURL ECVAM. RhE models use normal (*e.g.* non-transformed) human keratinocytes that, during culturing, form a multi-layered epidermis including a *stratum corneum* at the top, functioning as a barrier. The same RhE test methods, however using a different exposure protocol, are employed for skin corrosion testing. In addition, alternative methods for skin corrosion include the Transcutaneous Electrical Resistance (TER) assay, based on excised animal skin and the CORROSITEX assay, based on an *in vitro* protein matrix.
Table 3 provides an overview of those test methods currently accepted by regulators for standardised use. Please note that the use of OECD test guidelines is subject to specific requirements that may exist in some OECD member countries. The table moreover indicates those standardised testing methods that are currently undergoing update in view of adaptation to technical progress, amendment of performance standards or additional non-validation data on predictive capacity (see 2.2.2 for details). Briefly, these updates currently concern a number of OECD TGs, namely OECD TG 430 (addition of Performance Standards), TG 431 (extension of applicability domain to include also partial information on subcategorisation of corrosives) and TG 439 (inclusion of additional test method validated in reference to the TG's Performance Standards).

Table 3: EU test methods and OECD test guidelines for skin corrosion and irritation testing

Generic description of test method	Standardised description of test method in EU legislation (EC, 2008b)	OECD test guidelines
***In vivo* skin corrosion and irritation**		
In vivo Draize rabbit test for skin corrosion/irritation testing	**B.4** Acute toxicity: dermal irritation/corrosion	**TG 404** Acute Dermal Irritation/Corrosion
***In vitro* skin corrosion**		
Transcutaneous electrical resistance test (TER)	**B.40** *In vitro* skin corrosion: Transcutaneous electrical resistance test (TER)	**TG 430** *In vitro* skin corrosion: Transcutaneous electrical resistance test (TER) *> Updated version adopted by WNT in 2013*
RhE- Human Skin Models: Episkin™, Epiderm™, SkinEthic ™ EpiCS®(EST-1000)	**B.40bis** *In vitro* skin corrosion: human skin model test	**TG 431** *In vitro* skin corrosion: human skin model test *> Updated version adopted by WNT in 2013*
CORROSITEX®	***No EU test method available***	**TG 435** *In Vitro* Membrane Barrier Test Method for Skin Corrosion
***In vitro* skin irritation**		
In vitro skin irritation testing using RhE: Episkin™, Epiderm™ and SkinEthic ™ test methods	**B.46** *In Vitro* Skin Irritation: Reconstructed Human Epidermis (RHE) Model Test	**TG 439** *In Vitro* Skin Irritation: Reconstructed Human Epidermis Test Method *> Updated version adopted by WNT in 2013*

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2.3 Eye irritation

2.3.1 Brief description of the toxicological area

Eye irritation relates to adverse changes in the eye following the application of a test substance to its anterior surface which are fully reversible within 21 days of application (OECD TG 405). The EU has implemented the UN GHS for the classification and labelling of hazardous chemicals with Directive 1272/2008 (EU CLP) (EC, 2008). In brief, four representative *in vivo* endpoint(s) for final classification (corneal opacity, iritis, conjunctiva chemosis, conjunctiva redness) receive mean scores calculated following grading at 24, 48 and 72 hours after installation of the test material to the eye sack of three different animals. The test material is classified as 'Category 1' when at least in one animal the effects caused by the application of the test material are not expected to reverse, or have not fully reversed within an observation period of normally 21 days. Otherwise, if all effects have fully reversed within an observation period of normally 21 days, the test material is classified as 'Category 2'. UN GHS provides the option to distinguish 'Category 2' test materials into two optional subcategories (not implemented in EU CLP): 'Category 2A' (irritating to eyes) when the eye effects listed above are not fully reversible within seven days of observation; 'Category 2B' (mildly irritating to eyes) when the eye effects listed above are fully reversible within seven days of observation. Test materials that fall in none of the above two categories are not classified as eye irritants.

A number of OECD Test Guidelines based on *in vitro* methods related to the endpoint "Eye Irritation" already exist: TGs 437, 438, 460. In addition, in TG 405 a stepwise testing strategy is described for the determination of the eye irritation/corrosion properties of substances. Using this strategy, as well as a weight-of-evidence analysis (where all available information on the eye irritation potential is considered prior to proceeding to *in vivo* testing) is important to avoid the unnecessary use of animals. Reducing substance testing resulting in severe animal responses promotes both animal welfare and sound science. Although TG 405 is an *in vivo* test method, it also supports the 3 Rs principle in reduction and refinement of animal testing.

2.3.2 Development/optimisation/improvement of alternative methods

2.3.2.1 Development of testing strategies

It is generally accepted that, in the foreseeable future, no single *in vitro* eye irritation test will be able to replace the *in vivo* Draize eye test to predict across the full range of irritation for different chemical classes. However, strategic combinations of several alternative test methods within (tiered) testing strategies may be able to replace the Draize eye test. A possible conceptual framework for such (tiered) testing strategies was developed within an ECVAM workshop (Scott *et al.*, 2010). The framework is based on alternative eye irritation methods that vary in their capacity to detect either severe irritant chemicals (GHS 'Category 1') or chemicals considered non-irritant (GHS 'No Category'). According to this framework, the entire range of irritancy may be resolved by arranging tests in a tiered strategy that may be operated from either end: to detect first severe irritants and resolve absence of irritancy ("Top-Down Approach") or to proceed inversely, starting with the identification of non-irritants first ("Bottom-Up Approach"). Ocular irritant chemicals (GHS 'Category 2') will be resolved in a last tier in both approaches.

To evaluate the scientific validity of possible building blocks of such test strategies and to assess their possible placement within a Bottom-Up and Top-Down Approach, several validation studies of different *in vitro* methods were undertaken or are ongoing (see 2.3.3 below):

1. Organotypic *in vitro* assays: Bovine Corneal Opacity & Permeability test (BCOP), Isolated Chicken Eye test (ICE), Isolated Rabbit Eye test (IRE), Hens Egg Test on the Chorio-Allantoic Membrane (HET-CAM).
2. Cytotoxicity and cell function-based assays: Cytosensor Microphysiometer (CM), Fluorescein Leakage (FL), Neutral Red Release (NRR), Red Blood Cell test (RBC).
3. Reconstructed Human Tissue Models: EpiOcular™ EIT and SkinEthic™ HCE.

In the framework of a collaboration agreement between EURL ECVAM and Cosmetics Europe, analyses of *in vivo* Draize eye irritation data from three different chemical databases,were undertaken.. The objectives of such analyses were: 1) to evaluate the variability of the reference test (*i.e.*, the Draize eye irritation test), 2) to determine the most representative *in vivo* endpoint(s) for the final classification of a chemical (*e.g.* corneal opacity, iritis, conjunctiva chemosis, conjunctiva redness, days to clear) and 3) to determine the importance of recovery.
In addition, potential ways for combining *in vitro* tests methods in integrated testing strategies based on the concept of the Bottom-up and Top-down approaches have also been investigated..The analyses focused on how each method performs for each endpoint, combining data in an efficient way that enhances the predictive capacity of each method. It furthermore described the most optimal combinations of the different validated *in vitro* methods in testing strategies by using data mining techniques and statistical tools. Manuscripts describing the outcome of these studies are currently being prepared.

A promising method that has been developed to serve as a self-contained *in vitro* substitute for the Draize eye test is the *Ex Vivo* Eye Irritation Test (EVEIT). It combines improved biological relevance and enhanced process monitoring using in-time dynamic observation based on optical coherence tomography (OCT), thus allowing for quantification of endpoints covered by established organotypic *in vitro* assays such as corneal opacity and corneal swelling. EVEIT has proven able to analyse in detail structural damage and the progress of healing during several days, which is a sensitive tool to identify ocular irritants that cannot be otherwise classified by other *in vitro* methods. An appropriate operating procedure and prediction model to use the EVEIT in eye irritation testing is currently under development..

2.3.2.2 EURL ECVAM template for *in vivo* eye irritation classification

ECVAM developed a MS Excel-template for *in vivo* eye irritation classification of substances with existing Draize data to assist the development, optimisation and validation of alternative test methods or strategies (ECVAM, 2010).
The template and the accompanying explanatory document (Hoffmann *et al.*, 2010) are available for download on the EURL ECVAM website. The template includes built-in algorithms for calculating eye irritation classifications from Draize *in vivo* eye irritation data, according to the rules of EU DSD, EU CLP, UN GHS and of the United States Environmental Protection Agency (US EPA). The explanatory document provides detailed instructions on how to use the template.
EURL ECVAM recommends the use of this template to generate eye irritation classifications from existing reference *in vivo* Draize data for chemicals that shall be used in the development, optimisation and validation of alternative test methods or strategies for eye irritation.

2.3.3 Test Method Submissions

In the field of eye irritation, EURL ECVAM has received two test submissions in the time period between 2010 and 2012.

A cell function based *in vitro* test method that uses, beside cellular respiration and bioimpedance, changes in pH to characterise water soluble chemicals, was submitted in 2011. This test method evaluates the potential ocular toxicity of chemicals by measuring the reduction in the extracellular acidification rate of L929 cells treated by the test chemical. It is a similar test method to the validated test method based on the Cytosensor Microphysiometer. Since the test pre-submission was incomplete, additional information has been requested.

Another test method that predicts the ocular irritation potential was submitted in 2011. The test method mimics the biochemical phenomena of corneal proteins denaturation and disruption caused by irritant substances acting on the cornea. This is the second test method of the same kind that was submitted to EURL ECVAM. A first similar test method was submitted to ECVAM in 2009 and has undergone an external validation study that will be submitted to EURL ECVAM for evaluation and eventual ESAC peer review.

2.3.4 Validation of alternative methods

An Eye Irritation Validation Study (EIVS) on two Reconstructed human Tissue (RhT) test methods [EpiOcular™ Eye Irritation Test (EIT) and SkinEthic™ Human Reconstructed Corneal Epithelium (HCE)] coordinated by ECVAM and co-funded by Cosmetics Europe and ECVAM was initiated at the end of 2008 (Freeman *et al*., 2009).
EIVS assessed the validity of the SkinEthic™ HCE short-exposure (SE), long-exposure (LE) and test strategy and of the EpiOcular™ EIT protocols for liquids and solids as stand-alone (independent) test methods to reliably discriminate chemicals not classified as eye irritant ("non-irritants") from all other classes in the framework of a Bottom-Up approach (Scott *et al.,* 2010). The experimental part in which 104 coded chemicals (substances and chemical mixtures) were tested, finished in March 2012.

The outcome of the study is that both test methods are highly reproducible both in terms of within- and between-laboratory reproducibility.
For EpiOcular™ EIT, the protocol for the liquid chemicals met all the acceptance criteria of the Validation Management Group (VMG) for sensitivity, specificity and overall accuracy. On the other hand, not all of the acceptance criteria were met by the protocol for the solid chemicals. Analysis of the EIVS data for solids identified that there is scope for improvement. Therefore, the test method protocol for solids has undergone further optimisation.
For SkinEthic™ HCE SE, LE and testing strategy, not all of the acceptance criteria were met, neither using the short exposure (SE), nor the long exposure (LE), nor the strategy. Also for SkinEthic™ HCE, analysis of the data showed that there is scope for improvement and the test method protocol has undergone further optimisation.
The additional validation of the optimised EpiOcular™ EIT protocol for solids has already started whereas the SkinEthic™ HCE protocol optimisation requires more time and will therefore take place outside the scope of EIVS in order to avoid delays. The complete study is expected to be finalised within 2013.

2.3.5 Regulatory acceptance of alternative methods

In the area of eye irritation, four organotypic assays have previously been accepted by EU regulators for detecting severe ocular irritants (EC, 2004)..
OECD TGs based on cell-based methods (Fluorescein Leakage and Cytosensor Microphysiometer) were either already adopted in 2012 (TG 460) or currently being discussed within the WNT (draft TG on the Cytosensor Microphysiometer). The test methods have specific applicability domains and both of them can be used as an initial step

within a Top-Down approach to identify "ocular corrosives/severe irritants" (UN GHS/EU CLP category 1 or U.S. EPA category I). In addition, the Cytosensor Microhysiometer can also be used in a bottom-up approach to identify chemicals as "not classified for eye irritation" (UN GHS/EU CLP Not Classified or U.S. EPA category IV).
In addition, OECD TGs based on organotypic assays (Bovine Corneal Opacity and Permeability test, Isolated Chicken Eye test) were already adopted in 2009 (TG 437, TG 438). Both test methods have specific applicability domains and can be used as an initial step within a Top-Down approach to classify substances as "ocular corrosives and severe irritants" (UN GHS/EU CLP category 1 and U.S. EPA category I). In April 2013, updated test guidelines expanding the use of BCOP and ICE in a bottom-up approach to identify chemicals as "not classified for eye irritation" (UN GHS/EU CLP No Category) were adopted.

Table 4 contains the developments at OECD level related to TGs for in vitro eye irritation testing (2010-2013).

Table 4. Status of OECD TGs for *in vitro* eye irritation testing

Test Method	*OECD Test Guideline*	*Status (as of 31.12.2012)*
Cytosensor Microphysiometer (CM)	tbd	Draft proposal (12/2012)
Fluorescein Leakage (FL)	460	Adopted 10/2012
Bovine Corneal Opacity & Permeability test (BCOP)	437	Updated Test Guideline adopted (04/2013)
Isolated Chicken Eye test (ICE)	438	Updated Test Guideline adopted (04/2013)

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2.4 Genotoxicity and mutagenicity

2.4.1 Brief description of the toxicological area

Genetic alterations in somatic and germ cells are associated with serious health effects, which in principle may occur even at low exposure levels. Mutations in somatic cells may cause cancer if mutations occur in proto-oncogenes, tumour suppressor genes and/or DNA damage response genes, and are responsible for a variety of genetic diseases. Accumulation of DNA damage in somatic cells has also been proposed to play a role in degenerative conditions such as accelerated aging, immune dysfunction, cardiovascular and neurodegenerative diseases. Mutations in germ cells can lead to spontaneous abortions, infertility or heritable damage to the offspring and possibly to the subsequent generations.

For an adequate evaluation of the genotoxic potential three endpoints need to be assessed: gene mutation, structural chromosome aberrations, and numerical chromosome aberrations. Therefore, a battery of tests is needed for an adequate coverage of all endpoints. Although several *in vitro* tests for genotoxicity assessment are available, at the current status these are not sufficient to fully replace the animal tests needed to confirm the safety of cosmetics ingredients (Adler *et al.*, 2011).

2.4.2 Development/optimisation/improvement of alternative methods

The high false positive rate of the established *in vitro* genotoxicity tests leads to an increased number of follow-up *in vivo* tests that would be needed for the confirmation of these results. To address this issue, ECVAM organised a workshop in April 2006. The workshop aimed at discussing how to improve current *in vitro* tests and at reviewing the development of new tests. The recommendations of this workshop (Kirkland *et al.*, 2007) have paved the way for several international initiatives (*e.g.* of Cosmetics Europe, ILSI/HESI, EURL ECVAM, NC3Rs, JaCVAM).

Following one of the workshop recommendations, ECVAM coordinated an expert group that established lists of genotoxic and non-genotoxic chemicals recommended for the assessment of the performance of new and improved genotoxicity tests (Kirkland *et al.*, 2008). This chemical list is used world-wide by test developers and other organisations. Testing at high concentrations was identified as one possible source of false positives. To address this issue, an analysis of published data for top concentration considerations in mammalian cell genotoxicity testing was carried out which suggested that the top concentration could be reduced without any loss of sensitivity in detecting rodent carcinogens (Parry *et al.*, 2010).

2.4.3 Test Method Submissions

A pre-submission on a genomics-genotox assay was received in 2010. This is a transcriptomics-based *in vitro* test intended to accurately predict the *in vivo* genotoxic properties of chemicals.

Based on Ames-test data, the compounds are first stratified as Ames-positive or Ames-negative. Next, the expression changes induced by the compounds in HepG2 cells are compared with a large transcriptomics database for known Ames-positive/Ames-negative and *in vivo* genotoxic/non-genotoxic agents using a supervised clustering algorithm, in order to predict the *in vivo* genotoxic property of the compounds. The test method is biologically and mechanistically relevant to judge genotoxicity effects, however some limitations of the test may be related to the cell model employed, which may lack a complete metabolic active system. The full submission has recently been received and is currently under evaluation.

2.4.4 Validation of alternative methods

EURL ECVAM has been a partner in a project led by Cosmetics Europe, which aimed to establish and validate new methods for genotoxicity testing in reconstructed human 3D skin models (micronucleus test and comet assay) (Aardema *et al.*, 2010). Both tests are still under evaluation. The comet assay is now being validated in a joint effort between Cosmetics Europe and the German Federal Institute for Risk Assessment (BfR).

2.4.5 EURL ECVAM strategy in the area of genotoxicity

In order to achieve full replacement of regulatory animal testing for genotoxicity, it is necessary to improve the *in vitro* testing strategy. This will require: identification of *in vitro* false or misleading positives, identification of additional *in vitro* tests to follow-up *in vitro* positives, improvement of current tests and the development of novel tests. To start answering some of these questions, EURL ECVAM has organised in January 2013 a workshop on "How can *in vitro* mammalian cell genotoxicity tests reduce the need for *in vivo* follow-up testing with compounds positive in the Ames test". The driving hypothesis was triggered by a study recently conducted where it appeared that Ames-positive results associated with two negative *in vitro* mammalian cell tests may be predictive of non-genotoxic activity *in vivo* and non-carcinogenicity (D. Kirkland, personal communication). The aim of the workshop was to explore if there are any general trends that can be concluded from preliminary analyses of different (public and proprietary) databases regarding the "risk" predicted by different patterns of *in vitro* results. The workshop brought together world-wide renowned experts from regulatory agencies, different industrial sectors and academia (report in preparation). The preliminary results presented showed some consistency of patterns across databases which was considered promising. Given that such an exercise was recognised to have strong implications at regulatory levels, EURL ECVAM is committed to follow up on this.

EURL ECVAM has been involved in the drafting of the EFSA scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment, which took into consideration the need to avoid unnecessary animal tests (EFSA, 2011). In this opinion, as well as in other guidance documents the *in vitro* micronucleus test, which has previously been validated by EURL ECVAM (Corvi *et al*, 2008), has been recommended as the main *in vitro* test to be used in a two-test battery.

2.4.6 Regulatory acceptance of alternative methods

The OECD is currently revising all TGs related to genotoxicity. Recommendations developed in the ECVAM workshop on false positives and follow-up work have been taken into account in the draft revised TGs, as well as in the revision of the ICH Guidance of genotoxicity testing and data interpretation for pharmaceuticals (ICH, 2009). The revised TG will enhance the quality of the data that will be produced and consequently will avoid in some cases the need for follow-up confirmation of the results.

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2.5 UV-induced effects (Phototoxicity)

2.5.1 Brief description of the toxicological area

Phototoxicity (photoirritation) is defined as a toxic response that is elicited after the initial exposure of skin to certain chemicals and subsequent exposure to light, or that is induced by skin irradiation after systemic administration (oral, intravenous) of a chemical substance (Spielmann *et al.*, 1994). If a chemical absorbs UV or visible light, it needs to be determined if it is likely to cause adverse phototoxic effects when intended for human use.

2.5.2 Development/optimisation/improvement of alternative methods

2.5.2.1 3T3-NRU-PT assay

The 3T3-NRU-PT assay consists of the immortalised mouse fibroblast cell line, Balb/c 3T3 and is based on a comparison of the cytotoxicity of a chemical when tested in the presence and in the absence of exposure to a non-cytotoxic dose of simulated solar light. Cytotoxicity in this test is expressed as a concentration-dependent reduction of the uptake of the vital dye Neutral Red when measured 24 hours after treatment with the test chemical and irradiation (Borenfreund & Puerner, 1985). The test chemical together with the irradiation may alter the cell surface and in effect may result in a decreased uptake and binding of the Neutral Red Dye. Differences in this uptake can be measured with a spectrophotometer, which allows in essence the distinction and quantification between viable, damaged or dead cells.

One limiting factor of the 3T3-NRU-PT *in vitro* phototoxicity test is the requirement of aqueous solubility of the test substance. Reconstructed 3D human skin models could offer some advantages in comparison to the 3T3-NRU-PT:

- The 3D models have both viable primary skin cells and skin barrier, therefore they are directly relevant to the target tissue.
- A wide selection of chemicals, even in complex mixtures or in dermatological patches, can be applied, simulating more closely a topical application to the skin.
- Materials with extreme pH values can be tested.
- Histological comparison between control and exposed samples can be conducted.
- Depending on the barrier function of the stratum corneum, the absorption and penetration of the original chemicals or molecules created during exposure could provide more relevant results than tests performed on simpler systems (giving fewer false positive results).

2.5.2.2 EPIDERM™ Phototoxicity Test

In 1999 ECVAM funded a prevalidation study on the EpiDerm™ phototoxicity test, with promising outcome (Liebsch *et al.*, 1999). This model is based on the *in vitro* skin model Skin^2 (Edwards *et al.*, 1994) which had been discontinued in October 1996. It was further proposed by industry to apply the model in a tiered strategy to identify those chemicals that are predicted to be likely phototoxic in the 3T3-NRU-PT but are negative *in vivo* (Jones *et al.*, 1999). In response, the European Medicines Agency (EMA) has suggested in a Note for Guidance (NfG) on photosafety testing (CPMP/SWP/398/01), that confirmatory testing can be performed on such a skin model.

A feasibility study on the prevalidated human 3D skin model EpiDerm™-PT applied in phototoxic potency testing, demonstrated the usefulness of reconstructed human tissue models for prediction of phototoxicity of topically applied substances and formulations. In certain cases, this study demonstrated that the human condition may be underpredicted and that a precautionary factor of about 10 should be considered for extrapolation (Kejlová *et al.*, 2007).

2.5.2.3 Joint ECVAM-EFPIA Workshop on Phototoxicity

During this workshop held in 2010, experts from academia, the pharmaceutical industry and regulatory authorities presented 'hands-on' experience with the 3T3 NRU-PT in order to discuss why some results obtained in the pharmaceutical industry differ from the original validation exercise. The workshop was also organised to review technical issues and to reflect on strategies how to improve the usability of the assay for non-topical pharmaceuticals. (Cheridono *et al.,* 2012)

It became apparent, that changes to the protocol as outlined in the OECD Test Guideline 432 and the use of different sets of test chemicals are the main causes for the different test outcomes in comparison to the original validation study.

Furthermore, it was suggested that a reduction in the high number of positive results could be achieved

- by testing only relevant chemicals that have a Molar Extinction Coefficient (MEC) larger than 1000 L mol^{-1} cm^{-1},
- by limiting the maximum concentration under irradiation to 100 µg/mL and
- by considering higher concentrations without irradiation to assess IC_{50} values for Photo Irritation Factor (PIF) concentrations, if necessary.

Additionally, reconstructed human tissue models were proposed to be reliably applicable for the identification of phototoxic chemicals as an alternative to the 3T3 NRU-PT or in a second tier to confirm 3T3 NRU-PT positive results for the reduction in number of false positives (Cheridono *et al.,* 2012).

2.5.3 Test Method Submissions

No submissions in the area of phototoxicity testing were received at EURL ECVAM in the period of 2010 to first quarter of 2013.

2.5.4 Validation of alternative methods

The Japanese Center for the Validation of Alternative Methods (JacVAM) has coordinated and sponsored a validation study for the "Reactive Oxygen Species (ROS)-Assay". This test method is cell-free and based on the quantification of production of reactive oxygen species ("ROS", *i.e.* singlet oxygen and superoxide), generated from photo-irradiated chemicals (Onoue *et al.,* 2008). The results of this validation study have been published in April 2012 (Onoue *et al.,* 2012).

2.5.5 Regulatory acceptance of alternative methods

The 3T3-NRU-PT assay gained regulatory acceptance in all EU Member States in 2000 (see B.41 in EC, 2008) and was adopted as TG 432 by the OECD. It is now widely used in the chemical, pharmaceutical and cosmetics industries.

An increasing amount of data and experiences with regulatory photosafety testing over the past years have shown some severe shortcomings in the current guideline recommendations. In January 2008 the Committee for Medicinal Products for Human Use (CPMP) released a Concept paper (EMEA/534549/2007) commenting on the existing guideline on photosafety testing and suggesting ways to overcome the identified weaknesses.

Meanwhile ICH has decided to include photosafety testing as a new topic in the ICH framework (EMA, 2012). The newly formed ICH S10 will also follow-up on the 2010 workshop proposals to better define how data based on OECD TG 432 can be used for risk assessment of pharmaceuticals.

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3. Toxicological areas falling under the 2013 marketing ban

3.1 Repeated dose toxicity

3.1.1 Brief description of the toxicological area

Repeated dose toxicity studies in animals are carried out with the purpose of characterising the toxicological profile of a test substance in a mammalian species following daily administration of low doses for a defined period of time up to the whole lifespan of the animals (*i.e.* sub-acute, sub-chronic and chronic exposures). These tests provide information on possible adverse effects on target organs, on dose-response relationships, and on the reversibility/irreversibility of the effects. In addition, they offer the opportunity to demonstrate delayed and accumulative effects. Changes in many organs and tissues are considered together with clinical signs, clinical chemistry and haematology and, therefore, information on a wide range of endpoints is provided. The ultimate aim of these *in vivo* tests is to deliver the no-observed-adverse-effect level (NOAEL) or benchmark doses which are needed for extrapolation for risk assessment in humans (ECHA, 2012). These studies are described in a number of the OECD test guidelines including repeat dose 28 days oral, dermal and inhalation studies in rodents, 90 days oral, dermal and inhalation studies in rodents, 90 days and 1 year oral studies in non-rodents, and chronic toxicity studies in rodents (OECD TGs 407-413, TG 452; OECD, 1981a,b; OECD, 1998a,b; OECD, 2008; OECD, 2009a-c).

At present our knowledge of the many mechanisms leading to toxicity, the relevant pathways, their interaction and the impact on human health of some disturbances seen *in vitro* (*i.e.* biochemical changes, up and down regulation of genes) is limited. In a recent review Jennings *et al.*, (2013) summarised the major toxicologically relevant transcription factor-governed molecular pathways and described how toxins can directly or indirectly produce distinct gene expression bio-signatures.

3.1.2 Development/optimisation/improvement of alternative methods

An FP7 Research Initiative focusing on repeated dose toxicity was launched in 2011. This Research Initiative is a first step to addressing the long term strategic target of "Safety Evaluation Ultimately Replacing Animal Testing (SEURAT)". It is called "SEURAT-1", indicating that more steps have to be taken before the final goal will be reached.
SEURAT-1 (http://www.seurat-1.eu/) is co-funded by the European Commission under the FP7 Health theme and by Cosmetics Europe, the European Cosmetic Industry trade association. It consists of a cluster of seven projects: five complementary research projects, a central data management and servicing project (ToxBank), and a coordination and support action (COACH). The research projects relate to:

- "Stem Cells for Relevant Efficient Extended and Normalised Toxicology" (SCR&Tox)[8],
- "Hepatic Microfluidic Bioreactor (HeMiBio)[9]
- "Detection of endpoints and biomarkers of repeated dose toxicity using *in vitro* systems" (DETECTIVE)[10]
- "Integrated *In Silico* Models for the Prediction of Human Repeated Dose Toxicity of COSMetics to Optimise Safety" (COSMOS)[11]

[8] http://www.seurat-1.eu/pages/cluster-projects/scrtox.php
[9] http://www.seurat-1.eu/pages/cluster-projects/hemibio.php
[10] http://www.seurat-1.eu/pages/cluster-projects/detective.php
[11] http://www.seurat-1.eu/pages/cluster-projects/cosmos.php

- "Predicting long-term toxic effects using computer models based on systems characterization of organotypic cultures " (NOTOX)[12]
- "Supporting Integrated Data Analysis and Servicing of Alternative Testing Methods in Toxicology" (ToxBank)[13]

EURL ECVAM is participating in three scientific projects (DETECTIVE, Scr&Tox, COSMOS) and is also a driving partner of the overall coordination project (COACH). A Scientific Expert Panel (SEP) oversees progress and gives advice on strategy and scientific issues. All the projects started their activities in January 2011 and the complete initiative has a duration of six years.

The SEURAT-1 strategy is to adopt a toxicological mode of action (MoA) framework to describe how any substance may adversely affect human health, and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure needed for safety assessment. The SEURAT-1 is building up a series of Proof of Concepts to demonstrate that the strategy and tools can be applied in real safety assessment scenarios.

Cross-project efforts are undertaken to capture in a systematic manner our understanding of the biological mechanisms of repeated dose toxicity. The predictive framework being developed, the approach is based on a series of key events, each of which can be modelled separately by *in silico* or *in vitro* methods, with a view to integrating the resulting information into MoA pathways and Adverse Outcome Pathways (AOPs).

Within the SEURAT-1 consortium, the COSMOS project (http://www.cosmostox.eu/) is developing publicly available computational workflows based on the integrated use of open-access and open-source models for the prediction of repeated dose toxicity (Anzali *et al.*, 2012). This includes: a) the establishment of an inventory of cosmetic substances (including identifiers and chemical structures) and a repeat dose toxicity database (including oral and dermal data); b) the development of novel ways of establishing thresholds of toxicological concern (TTC), based on innovative chemistry based prediction approaches and PBTK/PBTD modelling. The applicability of the current TTC approach to cosmetics has also been demonstrated (Worth *et al.*, 2012).

Beside SEURAT-1, another EU FP7 project called Predict-IV (Profiling the toxicity of new drugs: a non-animal-based approach integrating toxico-dynamics and biokinetics; 2008-2013) aims to develop strategies to improve the assessment of drug safety in the early stage of development and late discovery phase, by an intelligent combination of non-animal-based test systems, cell biology, mechanistic toxicology and *in silico* modelling (http://www.predict-iv.toxi.uni-wuerzburg.de/). The dynamics and kinetics of cellular responses to toxic effects *in vitro* will be characterised. Three target organs, liver, kidney and central nervous system are taken into account. Overall, the approach taken consists in integrating biological effects with toxicokinetics and modelling ensuring the generation of real exposure data linked to effects. Recently, Wilmes *et al.*, (2012) have shown the benefits of integrating transcriptomics, proteomics and metabolomics together with pharmacokinetics in drug safety, using human renal epithelial cells (RPTEC/TERT1) exposed to the nephrotoxin Cyclosporine A in repeat dose. Although Predict-IV relates primarily to drug safety, the assays could potentially also be profitable to the cosmetics sector.

[12] http://www.seurat-1.eu/pages/cluster-projects/notox.php
[13] http://www.seurat-1.eu/pages/cluster-projects/toxbank.php

3.1.3 Test method submissions, validation of alternative methods and regulatory acceptance

No test methods have been submitted to EURL ECVAM for repeated dose toxicity testing. Considering the scientific challenges for the development of alternatives in this area, no methods are expected within the short and mid-term and consequently no validation studies are planned for the moment.

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3.2 Toxicokinetics

3.2.1 Brief description of toxicological area

Toxicokinetics, which describes the processes of Absorption, Distribution, Metabolism and Excretion (ADME) of a chemical, provides information on the time-dependent blood/plasma or tissue concentrations of a chemical and its potential for accumulation and biotransformation (*e.g.* metabolism). ADME processes provide also information on the potential for induction or inhibition of biotransformation as a result of exposure to the chemical entity. Metabolism has been considered a bottleneck for *in vitro* systemic toxicity, however cell systems which closely mimic the metabolism in human liver, providing more reliable data, are nowadays available.

Although OECD guidelines have been formulated for animal-based experiments for risk assessment there is increasing evidence that animal data are not reliable for extrapolation to human risk assessment, mainly due to species to species differences in physiology and toxicokinetic aspects (*e.g.* metabolism). For these reasons, and due to requirements in EU Directive 2010/63/EU on the protection of animals used for scientific purposes and the EU Cosmetic Regulation (EC 1223/2009), there is an increasing pressure to develop alternative (non-animal) methods, in particular in the toxicokinetic area.

When moving from the classical toxicological safety assessment based on the whole animal methods to approaches based on *in vitro* and *in silico* methods, toxicokinetics is perceived as a key element to assess systemic effects (Pelkonen *et al.,* 2012; Coecke *et al.,* 2012; Adler *et al.,* 2011).

3.2.2 Development/optimisation/improvement of alternative methods

3.2.2.1 Integration of toxicokinetic modelling into the prediction of *in vivo* dose-response curves without animal experiments

Computational (*in silico*) methods for toxicity prediction include approaches for grouping chemicals based on chemical and biological similarity (category formation), read-across of properties between chemicals within groups, (quantitative) structure-activity relationships ((Q)SARs) and expert (knowledge-based) systems. Additional types of computational methods include Physiologically-Based Toxicokinetic (PBTK) models, which can be used to extrapolate from known external exposure levels to internal concentrations at the target organ level. In addition, Physiologically-Based Toxicodynamic (PBTD) models can be used to simulate the internal concentration-response relationships of chemicals, based on the modelling of *in vitro* data. The coupling of PBTK and PBTD models is a relatively new approach which can be used to extrapolate from effective (toxicologically relevant) internal concentrations to external doses (*in vitro*-to-*in vivo* extrapolation; IVIVE).

In October 2011, the "EPAA/DG JRC ADME expert group organised a PBTK Workshop on "Potential for further integration of toxicokinetic modelling into the prediction of *in vivo* dose-response curves without animal experiments" brought together 50 experts in the field. A detailed manuscript is in preparation (Bessems *et al.,* 2013). The main aspects that will be detailed in the manuscript are related to the:

- Identification of gaps in non-animal test methodology for the assessment of ADME.
- Collection of models allocated to three stages of development where stage 1 is regarded as assay protocols that are suitable as input for PBTK modelling and ready for validation.
- Review of the availability of user-friendly PBTK software tools and free-to-use web applications.
- Understanding the requirements for wider and increased take up and use of PBTK modelling by regulators, risk assessors and toxicologists in general.

- Tackling the aspect of obtaining *in vivo* human toxicokinetic reference data via microdosing following the increased interest by the research community, regulators and politicians.

The next step in the area of toxicokinetics is the follow-up of specific recommendations and to consolidate efforts amongst all stakeholders. Some important aspects, which need attention in the immediate future and are based on current toxicokinetic discussions at the European level, can be listed as follows:

- To respond to today's specific regulatory demands for sector-specific regulations (*e.g.* to start using toxicokinetic *in vitro* and *in silico* methods for route to route extrapolation, to calculate bio-accumulative potential, waiving, read-across *etc.*).
- To use available toxicokinetic *in vitro* and *in silico* methods in high priority areas (*e.g.* skin sensitisation) for information needs on bioavailability.
- To use PBTK models to integrate rather isolated information on ADME to estimate the internal/tissue dose and use this information in design of the *in vitro* experiments.
- To use PBTK models for converting *in vitro* results (generated at tissue/cell or sub-cellular level) into dose-response information for the entire organism.
- To extend the first pilot version of the database ECVAM KinParDB (see http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/validation-regulatory-acceptance/systemic-toxicity/toxicokinetics) containing human and rat kinetic parameters (mainly based on intravenous and oral administration) for 100 chemical substances following assessment of their reliability which are required to build PBTK models.

3.2.2.2 Development of a predictive tool for human bioaccumulation assessment

Potential bioaccumulation of a chemical in humans is another key element in risk assessment but no models are nowadays available for bioaccumulation which takes into consideration the biotransformation potential of a chemical in the human organism.

A generic PBTK model has been developed which, based on human *in vitro* liver metabolism data, minimal renal excretion and a chronic exposure, is able to assess the bioaccumulative potential of a chemical. The approach has been analysed using literature data on well-known bioaccumulative compounds [*i.e.* persistent organic pollutants such as polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethane) (DDT), perfluorooctane sulfonate (PFOS)], liver metabolism data from Pelkonen *et al.*, (2009) and a subset of the ToxCast phase I chemical library. In total, 94 compounds including pharmaceuticals, plant protection products and industrial chemicals were evaluated. The results suggested that, human potential bioaccumulation can be assessed with two *in vitro* tests: one aimed at calculating the chemical binding to plasma proteins and the other at estimating the liver clearance by *in vitro* measuring the metabolite formation and/or the substrate depletion of the compound using human hepatocytes.

Both tests are suited for high-throughput analysis and thus can be used for prioritisations of chemical safety assessment (Tonnelier *et al.*, 2012).

3.2.2.3 Provision of a three dimensional metabolic test system for toxicokinetic and toxicodynamic applications

A pilot study used cultured HepaRG cells as three dimensional metabolic competent structures in a spinner-bioreactor (Leite *et al.*, 2012). The use of a cost-effective commercially available bioreactor, which is compatible with high-throughput cell analysis, constitutes an attractive approach for routine application. In order to assess specific aspects of the biotransformation capacity of the bioreactor-based HepaRG® system, the induction of the cytochrome P450 (CYP) 1A2, CYP 2B6, CYP 2C9, and CYP 3A4 enzymes and the activity of the phase II enzyme, uridine diphosphate glucuronoltransferase, were tested. The long-term functionality of the system was demonstrated by 7-week stable profiles of albumin

secretion, CYP3A4 induction, and uridine diphosphate glucuronoltransferase activities. Immunofluorescence-based staining showed formation of tissue-like arrangements including bile canaliculi-like structures and polar distribution of transporters. The approach is a good strategy to reduce the time necessary for obtaining fully differentiated cell cultures. Furthermore, HepaRG® cells cultured in three dimensional spinner-bioreactors are an attractive tool for toxicological studies, showing a liver-like performance and demonstrating a practical applicability for toxicokinetic and toxicodynamic approaches.

3.2.2.4 Open-source models for the prediction of repeated dose toxicity (SEURAT-1 consortium)

Within SEURAT-1, the COSMOS project is developing publicly available computational workflows based on the integrated use of open-access and open-source models for the prediction of repeated dose toxicity (Anzali *et al.*, 2012; see also chapter 3.1. on repeated dose toxicity). This includes, among others, the development of novel ways of establishing thresholds of toxicological concern (TTC), based on innovative chemistry based prediction approaches and PBBK/PBTD modelling. The applicability of the current TTC approach to cosmetics has also been demonstrated (Worth *et al.*, 2012).

3.2.2.5 Combination of *in vitro* and *in silico* methods to predict target organ effects on humans under repeated dose exposure

Within the COSMOS project, research is ongoing to provide case studies for selected chemicals that illustrate how *in vitro* and *in silico* methods can be combined to predict target organ effects on humans under repeated dose exposure. This includes the development of QSARs for skin penetration, as well as mathematical models for route-to-route extrapolation and IVIVE. A virtual cell-based assay model has been developed to simulate the kinetics and dynamics of chemical compounds in cell-based assays (Zaldívar *et al.*, 2012). In parallel, models have been developed to simulate kinetics and dynamics at the organ level (virtual liver) and whole organism level (PBTK) models for the rat and the human. Furthermore, the models are being integrated in a multi-scale modelling approach, thereby coupling hepatocytes, via the virtual liver, to the whole organism (Diaz Ochoa *et al.*, 2013).

3.2.3 Test Method Submissions

A Skin Parallel Artificial Membrane Permeability Assay (PAMPA) was submitted to EURL ECVAM in 2012. Skin PAMPA, developed in 1998, is an *in vitro* assay screening for ADME and mainly for passive, transcellular permeation (Kansy *et al.*, 1998). It is the newest test in this line of *in vitro* permeability methods. With no requirements for human/animal skin tissue, Skin PAMPA employs an artificial membrane specifically designed to mimic the human stratum corneum (SC), the major barrier of skin. It contains a synthetic structural analogue similar to the ceramide component of the skin-lipid matrix. The other major lipid matrix components (cholesterol and free fatty acids) are also included (Sinkó *et al.*, 2012).
Skin PAMPA data are currently being analysed within a cosmetics decision context for oral to dermal extrapolation in the framework of the SEURAT-1 Project COSMOS. The permeability or penetration rate is determined by quantitation of the fraction of the test compound found in the receiver compartment after a period of incubation using UV-Vis spectrophotometry, HPLC or LC/MS, among other suitable analytical methods. In comparison to the *in vitro* human skin permeability data, Skin PAMPA allows a faster throughput and measures only passive permeability, which can be also seen as an advantage depending on the regulatory use context. The evaluation by EURL ECVAM of the pre-submission on the test method in subject is on-going.

3.2.4 Validation of alternative methods

At a stakeholder meeting in the field of toxicokinetics and metabolism, it was agreed that a validation study providing a standard for human hepatic metabolism and toxicity would have been very beneficial. As a follow-up, EURL ECVAM coordinates the "Multi-study Validation Trial for cytochrome P450 (CYP) induction providing a reliable human-metabolic competent standard method using the human cryopreserved HepaRG® cell line and cryopreserved human hepatocytes". In September 2010 and in October 2012 two meetings took place on the progresses of the CYP induction validation project involving all the test facilities and the Validation Management Group.

The CYP induction assay is based on either cryopreserved human hepatocytes or a cryopreserved human metabolically competent cell line (HepaRG®) and is standardised using coded test items. The coded test items are selected on the basis of evidence from *in vivo* human data on their CYP induction potential. Furthermore, the chemical selection took also into consideration that all three main nuclear receptors [pregnane-X-receptor (PXR), Ah receptor (AhR) and the constitutive androstane receptor (CAR)] involved in CYP induction are covered. The endpoint "induction of CYPs" is measured following treatment with test compounds and two reference compounds (ß-naphthoflavone and rifampicin), using a cocktail of prototypical substrates for different CYP isoforms incubated directly with the two test systems. The EURL ECVAM GLP Test Facility is participating with its own laboratories in this study.

The objectives of the validation study are the following:

- To provide a reliable test system for toxicokinetics and toxicodynamics applications. The metabolic competence of the two test systems is assessed by measuring the potential of prototypical inducers to induce CYP enzymes. CYP induction, indeed, having a complex underlying mechanisms (gene activation by xenobiotic-sensing nuclear receptors, followed by *de novo* protein synthesis) is a good endpoint to assess high quality metabolic competence.
- To assess the longer term (compared to previous test systems) metabolic competence of two test systems. HepaRG® cells could be used as a long term cellular system for metabolism of drugs and other xenobiotics with a low turnover. These substances are notoriously difficult to study in current systems using primary human hepatocytes, because of short viability and stability.
- To assess a preliminary predictive capacity of the test systems for the potential of selected test items to induce CYP enzymes. The four CYP isoforms have been selected as the main CYP enzymes involved in the metabolisms of drugs and xenobiotic and covering all the three main receptors (CAR, AhR, PXR) involved in the xenobiotic-receptor binding which triggers CYP induction. Both FDA and EMA Guidelines recommend CYP1A2, CYP2B6, CYP2C9 and CYP3A4 for CYP induction studies of new drug candidates to understand the impact on kinetics of both the new compound itself and on possible co-medications.

In addition in moving towards a mode of action based approach to safety assessment, up-regulation of CYP iso-enzymes has been identified as a key event potentially leading to a number of adverse events and as such this assay may contribute to the elucidation of a number of adverse outcome pathways.

The experimental phase of the cryopreserved HepaRG® test system of the CYP induction assay has been finalised. Completion of the whole study is foreseen by the end of spring 2013, with the ESAC peer review starting in October 2013.

3.2.5 Regulatory acceptance of alternative methods

Following the good experimental results in terms of reliability and reproducibility, presented at the last Validation Management Group (VMG) meeting of the CYP-induction validation study (October 2012), a Standard Project Submission Form (SPSF) for the development of a Performance-based Test Guideline (PBTG) on the establishment of human-derived hepatic system to investigate biotransformation and toxicity of compounds, by evaluation of cytochrome P450 induction competence, has been submitted to OECD in December 2012 and was taken up in the OECD work programme in April 2013..

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3.3 Skin sensitisation

3.3.1 Brief description of the toxicological area

Skin sensitisation is the toxicological endpoint associated with substances that are able to elicit an allergic response following contact with the skin, termed allergic contact dermatitis in humans. Sensitisation evolves in two phases: the first phase is the induction of specialised immunological memory in an individual following exposure to an allergen. The second phase is elicitation, *i.e.* the production of a cell-mediated allergic response by exposure of a sensitised individual to the same allergen. Skin sensitisation represents an important consumer safety endpoint for the cosmetics sector. The conduct of the risk assessment process for cosmetics ingredients relies on a good understanding of the relative potency of the sensitiser which is traditionally experimentally determined in animal models.

3.3.2 Development/optimisation/improvement of alternative methods

Important progress has been made in the development of alternative methods for this endpoint due to the good understanding of the biology and the chemistry at the basis of this toxicological effect, as recently documented in the Adverse Outcome Pathway (AOP) for skin sensitisation developed by the OECD (OECD, 2012a,b). Test methods under development/evaluation address key events of the skin sensitisation pathway. Given the complexity of the biological mechanisms underlying this human health effect, it is generally agreed that combinations of mechanistically-based test methods will be needed for both hazard assessment and potency prediction. Although it is expected that skin sensitisation hazard identification can be achieved in the short term without the use of animals, potency prediction remains more challenging (Adler *et al.*, 2011).

3.3.3 Test Method Submissions

Progress in the development of alternative methods for this endpoint is reflected by the numerous submissions received by EURL ECVAM in 2010-2012. These include test methods designed to address: peptide binding, inflammatory and oxidative stress responses in keratinocytes, gene or protein expression profiles in reconstructed human epidermis (RhE) or human dendritic cells (DC)-like cell lines, cytotoxicity in RhE models as expression of the irritant property of allergens, DC migration, histological damage in skin explants as a result of T cells activation. Test submission received by EURL ECVAM included proposals on the use of these methods in combination for hazard identification and/or potency predictions.

3.3.4 Validation of alternative methods

EURL ECVAM is currently evaluating mechanistically relevant test methods for their reliability (transferability, within and between laboratory reproducibility) and preliminary predictive capacity in view of their future use as part of integrated approaches for skin sensitisation hazard assessment and characterisation.

Test methods in advanced status of evaluation include 1) the Direct Peptide Reactivity Assay (DPRA), which uses HPLC to monitor a chemical's potential to deplete a nucleophile-containing synthetic peptide, 2) the human Cell Line Activation Test (h-CLAT) which measures the induction of protein markers, associated with DC maturation *in vivo*, on the surface of DC-like cell lines following exposure to the chemical and 3) the KeratinoSens in which the activity of the Nuclear factor-erythroid 2 (Nrf2) Antioxydant Response Element (ARE)-binding transcription factor in keratinocytes, following exposure to the chemical, is measured.

The DPRA and the KeratinoSens underwent independent peer review by the ESAC whereas the peer review process for the h-CLAT is expected to start in 2013. All of these methods appear to be adequately reproducible to worth further consideration as part of future

integrated non-animal approaches for skin sensitisation. In some cases quantitative information can be derived from these methods, nevertheless how such information can be used for potency prediction still remains to be fully determined.

3.3.5 EURL ECVAM strategy in the area of skin sensitisation[14]

In order to achieve full replacement of regulatory animal testing for skin sensitisation, potency information would need to be generated with non-animal approaches. EURL ECVAM recently made an assessment of the regulatory needs for this endpoint within pieces of EU legislation where the generation of skin sensitisation information represents a standard requirement. On the basis of this and with a view to achieve the biggest impact on the saving of animals, EURL ECVAM has decided to focus its efforts in the area for the next five years on the development of non-animal testing strategies suitable for the hazard identification and subcategorisation of sensitisers (subcategories 1A and 1B as adopted in the 3rd revised version of the Globally Harmonised System of Classification and Labelling of Chemicals) (GHS; UN, 2011) since this would satisfy the requirements of the majority of chemicals regulations in the EU and would contribute to a global approach for skin sensitisation testing. EURL ECVAM will also play a leading role within the OECD to develop a set of complementary Test Guidelines and related guidance documentation that will facilitate a globally accepted approach for skin sensitisation hazard identification and classification (Casati *et al.*, 2013). The availability of alternative methods able to identify the skin sensitisation hazard potential of chemicals with a sufficient level of accuracy would also contribute to meeting the safety assessment requirements of the Cosmetics Regulation since the risk assessment process requiring potency information is conducted only for those chemicals for which a sensitising hazard has been identified.

Against this background EURL ECVAM is initiating an in-house project on the development of integrated testing strategies (ITS) (based on combinations of *in silico*, *in chemico* and *in vitro* methods) for hazard identification and classification. The ultimate goal will be to propose scientific solutions that can satisfy the requirements of EU chemicals legislation, in particular the Classification Labelling and Packaging of substances and Mixture Regulation (EC, 2008) and REACH (EC, 2006) and that can be considered by the OECD for global implementation.

A complementary project is being conducted by the Cosmetics Europe Skin Tolerance Task Force aimed at the evaluation of the potential of *in vitro* test methods to contribute to potency prediction.

EURL ECVAM is partnering the European Partnership for Alternative Approaches to Animal Testing (EPAA) Platform 3Rs in Regulation. The main objective of the Platform is to improve the implementation of 3Rs in European regulatory testing and decision making. In the framework of this activity a Skin Sensitisation Training Workshop was held in February 2013 at ECHA, Helsinki. The purpose of the Workshop was to start engaging the regulators from ECHA and OECD in view of developing strategies for hazard identification and characterisation which use non-animal methods.

[14] EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification. JRC Scientific and Technical reports. Available at: http://publications.jrc.ec.europa.eu/repository/handle/111111111/27708. Accessed on 20 April 2013.

3.3.6 Regulatory acceptance of alternative methods

So far there are no regulatory accepted non-animal approaches for skin sensitisation. The OECD has already expressed interest in *in vitro* methods for skin sensitisation by including some of them (*i.e.* DPRA and KeratinoSens) in its 2012 work programme. In the third quarter of 2012 the European Commission/EURL ECVAM jointly with the Japanese Center for the Validation of Alternative Methods (JaCVAM) submitted a SPSF to the OECD Test Guidelines Programme for the development of a Test Guideline for the h-CLAT which was approved in April 2013.

Unlike test guidelines, Integrated Approaches to Testing and Assessment (IATA, i.e ITS) fall outside the scope of Mutual Acceptance of Data (MAD). Consequently there is a need to have a harmonised framework for the documentation and interpretation of results generated with such integrated approaches. Against this background in 2013, EURL ECVAM submitted a project proposal to the OECD to establish a drafting group with the mandate of developing a Guidance Document on the Evaluation and Application of IATA for skin sensitisation. The aim of this project, which will be run by the OECD Hazard Assessment Task Force, is to develop generic guidance on how to document and evaluate IATA in view of their intended applications to facilitate globally accepted approaches for the assessment of skin sensitisation.

3.3.7 References

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Casati S, Worth A, Amcoff P, Whelan M. (2013). EURL ECVAM Strategy for Replacement of Animal Testing for Skin Sensitisation Hazard Identification and Classification. Accessible at: http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/replacement-of-animal-testing-eurl-ecvam-releases-its-strategy-for-skin-sensitisation-hazard-identification-and-classification/.

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3.4 Carcinogenicity

3.4.1 Brief description of the toxicological area

Substances are defined as carcinogenic if after inhalation, ingestion, dermal application or injection they induce (malignant) tumours, increase their incidence or malignancy, or shorten the time of tumour occurrence. It is generally accepted that carcinogenesis is a multihit/ multi-step process from the transition of normal cells into cancer cells *via* a sequence of stages and complex biological interactions, strongly influenced by factors such as genetics, age, diet, environment, hormonal balance, *etc.* Since the induction of cancer involves genetic alterations which can be induced directly or indirectly, carcinogens have conventionally been divided into two categories according to their presumed mode of action: genotoxic carcinogens and non-genotoxic carcinogens. Genotoxic carcinogens have the ability to interact with DNA and/or the cellular apparatus (such as *e.g.* the spindle apparatus and topoisomerase enzymes) and thereby affect the integrity of the genome, whereas non-genotoxic carcinogens exert their carcinogenic effects through other mechanisms that do not involve direct alterations in DNA.

The complexity of the carcinogenicity process makes it difficult to develop *in vitro* alternative test models that mimic the full process, especially for non-genotoxic chemicals. The challenge in developing *in vitro* alternatives is also heightened because of the complexity of the number of target organs. It is expected that an integrated approach involving multiple *in vitro* models will be needed, but a better understanding of the entire process is necessary before this will be possible (Adler *et al.*, 2011). While *in vitro* and *in vivo* genotoxicity tests contribute to the assessment of genotoxic carcinogens, there is a lack of tests available for the assessment of non-genotoxic carcinogens.

3.4.2 Development/optimisation/improvement of alternative methods

The potential of new approaches such as 'omics'- technologies have been explored in the carcinoGENOMICS FP6 project. This project aimed at developing toxicogenomics- and metabolomics-based *in vitro* tests to detect potential genotoxicants and carcinogens (http://www.carcinogenomics.eu/). In the frame of the project two test methods have been selected for further optimisation: a toxicogenomics-based test in HepaRG cells for the liver and a toxicogenomics-based test in RPTEC/TERT1 cells for the kidney. The optimisation/prevalidation work package was coordinated by EURL ECVAM and aimed at 1) further developing these two test methods by testing 15 additional chemicals; 2) assessing test methods transferability and reproducibility using the same agreed Standard Operating Procedures and 3) develop dedicated bioinformatics tools to serve as basis for future validations of omics-based tests. Using different bioinformatics approaches both models were shown to be robust and reproducible. While the HepaRG model was shown to identify genotoxic carcinogens, the RPTEC/TERT1 model could reproducibly discriminate between the three classes of compounds tested: non-carcinogens, genotoxic and non-genotoxic carcinogens. Overall, these results present a proof of concept that such *in vitro* models can be used for transcriptomics analysis.

EURL ECVAM is also involved in the EPAA project on advancing the 3Rs in regulatory toxicology, where a workshop on "Carcinogenicity testing: scope for harmonisation and advancing the 3Rs" was held in Brussels on the 28th of February 2013.

3.4.3 Test Method Submissions

One test method has been submitted to assess genotoxicity (see chapter 2.4.3).

3.4.4 Validation of alternative methods

The *in vitro* cell transformation assays (CTAs) have been shown to closely model some key stages of the *in vivo* carcinogenesis process and have been in use for more than four decades to screen for potential carcinogenicity as well as investigate mechanisms of carcinogenicity. Moreover, CTAs are faster and more cost efficient than the *in vivo* rodent carcinogenicity assay, providing a useful approach for screening of chemicals with respect to their carcinogenic potential.

As with some other assays with a long history of use, CTAs had not undergone formal validation in accordance with current standards (OECD, 2005). Therefore, ECVAM coordinated an international study that was designed to address issues of CTA protocols standardisation, transferability and reproducibility. The study assessed to protocol variants for the SHE CTA (at pH 6.7 and pH 7.0) and the BALB/c 3T3 assay. This study was peer reviewed by the ESAC that issued an ESAC opinion, leading to the publication of a EURL ECVAM Recommendation on three CTAs for assessment of the carcinogenic potential of chemical substances (ECVAM, 2012).

The complete study results as well as the recommended CTA protocols and photo catalogues developed during the ECVAM study are published in a special issue of Mutation Research on CTA (Corvi and Vanparys, 2012). The SHE CTA protocol and the corresponding photo catalogues can also be downloaded from the EURL ECVAM DataBase service on ALternative Methods (DB-ALM) (http://ecvam-dbalm.jrc.ec.europa.eu/public_view_doc.cfm?id=59F6E6BF14DB487AB4B58FBDE3563BDE9CA32B2BCED0910E01C64222EAD76E61A3291B895581F634).

Another variant of CTA, the Bhas 42 CTA which is derived from the BALB/c 3T3 CTA, was validated by the Japanese Centre for the Validation of Alternative Methods. The study, which addressed two protocols (a 6-well and a 96-well method) was peer reviewed by ESAC in 2012.

In conjunction with other available data, CTAs can be used to determine whether it is necessary to carry out animal testing for carcinogenicity in the context of EU legislation.

3.4.5 EURL ECVAM strategy in the area of carcinogenicity

In order to achieve full replacement of regulatory animal testing for the assessment of carcinogenicity potential, approaches to assess both genotoxic and non-genotoxic carcinogens are needed. The EURL ECVAM strategy to improve testing for genotoxicity is outlined above. For non-genotoxic carcinogenicity assessment no strategy is in place. Despite the fact that some mechanisms behind non-genotoxic carcinogenicity are known, multiple unknown mechanisms of action and the insufficient knowledge of the cellular and molecular events have not yet allowed for the implementation of a battery of *in vitro* tests that could predict and/or explain their carcinogenic potential to humans.

3.4.6 Regulatory acceptance of alternative methods

Based on the EURL ECVAM protocols evaluation, an OECD Test Guideline on the CTA in SHE cells has been submitted to the WNT for discussion at its meeting in April 2013, The draft TG has not yet been adopted since some OECD Member Countries felt that there was a need to develop a Guidance Document first. CTAs are considered to provide additional useful information to more routinely employed tests for assessing carcinogenic potential and are therefore listed in various recent guidelines and testing strategies for such purposes (SCCS, 2010; Jacobson-Kram and Jacobs, 2005; ECHA, 2008; Pfuhler *et al*., 2010).

3.4.7 References

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3.5 Reproductive and developmental toxicity

3.5.1 Brief description of the toxicological area

Reproductive toxicity refers to a wide variety of adverse effects that may occur in different phases within the reproductive cycle, as a consequence of one or more exposures to a toxic substance, including effects on fertility, sexual behaviour, embryo implantation, embryonic/foetal development, parturition, postnatal adaptation, and subsequent growth and development into sexual maturity.

Among the various stages in the reproductive cycle, embryo-foetal development is considered as one of the most critical steps. Substantial effort has been spent in the development of promising alternative tests, such as tests based on the zebrafish embryo models and the pluripotent embryonic stem cell models, to allow for the detection of the teratogenic potential of substances. However, besides their current role as mechanistic support and screening tools, the role of alternative methods as part of integrated testing strategies for regulatory toxicity evaluations has to be defined further.

The complexity of mammalian reproduction requires integrated testing strategies to be able to fulfil all needs for hazard identification and risk assessment. A promising way forward is the use of recently established comprehensive databases in which toxicological information derived from standardised animal experimentations is collected.

3.5.2 Development/optimisation/improvement of alternative methods

A lot of efforts are on-going to optimise the embryonic stem cell test in particular for improving its predictions. However, more efforts are needed to understand which mode of actions can be picked up. Other areas using pluripotent stem cells for prediction of developmental (neuro) toxicity are being further developed for more specific applications (*e.g.* in the FP7 project "ESNATS").

Some substances may produce toxic effects by altering the function of the endocrine system. Concern for endocrine disrupting substances has led to specific provision for their identification and control under different pieces of legislation, including the cosmetic products regulation, which requires under Article 15 (4) that the regulation be reviewed as soon as community or internationally agreed criteria for endocrine disrupting substances become available.

The reproductive system and development of the foetus are considered particularly sensitive to toxicants acting by endocrine disrupting modes of action. Identification will require screening substances for endocrine activity. To this end *in vitro* assays are being developed to detect a range of different activities, such as the potential to interact with hormone receptors, which may be the initiating events for a number of different adverse effects *in vivo*. Such an approach fits very well and thus contributes to a mode of action based approach to understand how a substance may be toxic, an approach which is fundamental to the SEURAT strategy towards replacement of animal testing (see chapter 3.1.2).

3.5.3 Test Method Submissions

The Bovine Oocyte Maturation assay was submitted to EURL ECVAM in 2010. The test method that was developed within the framework of the FP7 project ReProTect is designed to cover aspects of human reproductive toxicity, in particular the maturation of mammalian oocytes. A summary of the submission was provided to the EURL ECVAM network of regulators [Preliminary Assessment of Regulatory Relevance (PARERE)] for assessing the regulatory relevance of the test method. Some regulators saw the value of the method as a component of an integrated testing strategy for reproductive toxicity at long term, as well as

a screening assay in short term, particularly under REACH, to identify potential reprotoxicants for further assessment. Whereas other regulators generally recommended that the place of such a method in a testing strategy and its impact on the 3Rs should be clarified first before any validation activities were undertaken. In the short term, the test method was seen as serving more to provide additional mode of action information without using animals but not reducing or replacing animal use in itself.

In addition, several test methods measuring the endocrine disrupting potential of chemicals have been submitted to EURL ECVAM in 2010. These were either binding assays (*i.e.* androgen receptor binding assay) or transcriptional assays measuring either (anti-)androgenic activity or (anti-)estrogenic activity, respectively. Some of the transcriptional assays for measuring the (anti-)androgenic activity were prioritised for validation at EURL ECVAM.

Finally, a genomic embryonic stem cell test was submitted to EURL ECVAM in 2012. The method addresses human embryotoxicity and consequently predicts developmental effects on the basis of alterations of gene expression of cell differentiation. The test method has not been prioritised for validation for the time being, the main reason for this being the lack of clarity how the test method is able to pick up crucial effects in the development of an adversity.

3.5.4 Validation of alternative methods

Tests for the detection of substances which can bind to estrogen or androgen receptors and activate (agonist) or block (antagonist) cellular responses are in the process of validation in studies led by EURL ECVAM. Once validated, these assays will contribute towards OECD performance-based test guidelines for estrogen receptor and androgen receptor transactivation assays.

An EURL ECVAM recommendation on a transactivation assay that detects chemicals with (anti-) estrogenic potential using a specific cell line is planned for 2013. This assay will feed into OECD TGs 455 and 457 (see below).

3.5.5 Regulatory acceptance of alternative methods

A performance-based test guideline for the detection of estrogen receptor agonists (TG 455) as well as a TG based on an assay for the detection of estrogen receptor antagonists (TG 457) were adopted by the OECD in 2012.

3.5.6 References

OECD (2012). Test No. 455: Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264185388-en

OECD (2012). Test No. 457: BG1Luc Estrogen Receptor Transactivation Test Method for Identifying Estrogen Receptor Agonists and Antagonists, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing. doi: 10.1787/9789264185395-en

4. Dissemination of Information

4.1 The EURL ECVAM DataBase service on ALternative Methods to animal experimentation (DB-ALM)

The DB-ALM [15] represents an important scientific instrument to implement the communication and dissemination strategy of the Joint Research Centre on animal alternatives as requested by the Commission and the European Parliament.[16]

The key feature of the DB-ALM is to provide user-oriented documentation in the form of quality controlled descriptions of alternative approaches prepared by experts and are, consequently, ready for immediate use (*factual* and *evaluated* information) representing an outcome of extensive bibliographic reviews and/or direct contacts with the method owners/users. Information at various levels of detail is provided to approach the scientific and regulatory community, as well as non-experts in the animal alternatives field. Current focus is given to non-animal methods in use for toxicity assessments of chemical compounds and/or formulations at all stages of development, validation or regulatory acceptance.

Method descriptions cover their objectives and applications, the scientific principle, including a discussion on their potential and eventual limitations completed with the status of development, validation or regulatory acceptance. These review documents are complemented with detailed technical descriptions in the form of protocols, where available, to allow the transfer of a technique to the laboratory.

Status

To date, the DB-ALM includes 154 method summaries, 138 protocols, 64 evaluations and details on 19 formal validation studies, as well as 8543 individual test results. It covers five topic areas in the form of thematic reviews while individual protocols are made available for 20 topic areas. To promote the exchange of information among people/institutions active in the animal alternatives field, the DB-ALM further manages a data sector on who's who in this field of science where information for over 200 registrations are made available.

The past three years have shown a further consolidation of the DB-ALM where the online information content has been enhanced and/or revised by 33% leading to a constant average and annual increase in new registrations to the service by 15% compared to the years before, and in total to over 2800 registered users from 82 countries covering representatives from academia (45%), industry (33%) and regulators (13%), the animal welfare movement and others (9 %).

In 2010, the European Partnership of Alternative Approaches to animal testing (EPAA) sponsored the Thematic Review Project on Reproductive Toxicity by using the DB-ALM. This was a milestone for achieving EPAA's major objective concerning the dissemination of information on available alternative methods. The thematic review has successfully been completed in 2012 leading to the update of an entire data sector of the DB-ALM, complemented by two surveys carried out within industry on the use of all 3Rs methods and strategies for reproductive toxicity testing.

In 2011, the AXLR8 coordination and support action (funded by DG Research & Innovation) that aims to accelerate the transition period versus a more sophisticated approach to chemical and product safety assessment, whilst reducing the animal use, indicated to the researcher and technology developers sponsored under the EU framework programmes, the DB-ALM as the platform where to store and disseminate their FP6 and FP7 research projects results regarding the development of non-animal methods.

[15] Access to DB-ALM: http://ecvam-dbalm.jrc.ec.europa.eu

[16] The DB-ALM originated from the Communication of the Commission to Council and European Parliament SEC (91)1794 and has further been reinforced by Directive 2010/63/EU.

4.2 EURL ECVAM Search Guide

The DB-ALM has been complemented by the EURL ECVAM Search Guide[17] that has specifically been developed to inform and support untrained database users to find high quality information on relevant alternative methods and strategies in an easy, yet systematic, and efficient way. This will be most relevant where regulatory requirements mandate the application of the 3Rs. The EURL ECVAM Search Guide provides search procedures, suggested search terms and user guidance to facilitate the location of the desired information on 3Rs animal alternatives in addition to an inventory of relevant information resources.

The EURL ECVAM Search Guide has first been published as a handbook in 2012. After one month of being in the public domain, it reached the 4th position in the Top 10 of the most downloaded publications of the EU Bookshop in June 2012. A re-edition is planned for spring 2013. The guide is also being developed as an Internet based application and is expected to be made available online in summer 2013.

[17] Access to EURL ECVAM Search Guide: http://bookshop.europa.eu/ - select: ECVAM Search Guide

5. International Cooperation

5.1 ICATM

EURL ECVAM together with its Japanese (JaCVAM), North American (US-ICCVAM and Health Canada) and Korean (KoCVAM) collaboration partners of the International Cooperation on Alternative Test Methods (ICATM) have significantly enhanced their collaborations in the period 2010 to 2012. ICATM has as its purpose to expand and strengthen collaboration, and communication among national validation organisations on the scientific validation and evaluation of new alternative testing methods proposed for regulatory health and safety assessments and to develop harmonised recommendations for their regulatory usability. EURL ECVAM is involved in ICATM collaborations in the areas of phototoxicity, eye irritation, skin sensitisation, endocrine disruption, toxicokinetics and carcinogenicity.

ICATM collaboration partners have met at least twice per year and the last meeting was held at JaCVAM, Ministry of Health, Labour and Welfare (MHLW), Tokyo, Japan on 13-14 February 2013 and the next scheduled meeting will be held at the XIII International Congress of Toxicology (KoCVAM) in Seoul, Korea on 1-4 July 2013. ICATM may possibly be expanded in 2013 to also include the Chinese National Institutes for Food and Drug Control (NIFDC) of the Chinese Food and Drug Administration (SFDA) as an observer organisation upon a showed interest from the Chinese authority to participate in upcoming meetings.

5.2 The "Tox21" initiative in the USA

The consortium "Tox21" is a collaboration based in the USA between the National Institute of Environmental Health Sciences/National Toxicology Program, the National Institutes of Health/National Centre for Translational Sciences (NCATS), the Environmental Protection Agency, and the Food and Drug Administration. The agencies work together to research, develop, validate and translate innovative chemical testing methods based primarily on *in vitro* methods that characterise toxicity pathways, as proposed in the National Research Council's landmark report (2007) entitled, "Toxicity Testing in the 21st Century". At the heart of the programme is the use of a robotic ultra-high throughput screening platform based at NCATS to repeatedly screen a chemical library comprising approximately 10,000 chemical substances, selected due to their known toxicity (controls) or because of concern in relation to their potential negative impact on human health or the environment.

The JRC/ECVAM has been collaborating closely with the Tox21 partners in the last few years and intends to get more engaged in the four thematic 'predictive toxicology' projects that have recently been devised within Tox21, namely, genotoxicity, cardiotoxicity, mitochondrial toxicity, and endocrine disruption. To support this closer engagement the JRC and NCATs will soon establish a formal Collaboration Agreement to facilitate in particular sharing of materials and data not yet released in the public domain.

5.3 ICCR

The International Cooperation of Cosmetics Regulations[18] (ICCR) is a four-lateral dialogue between the US Food and Drugs Administration, the European Commission, Health Canada and the Ministry of Health, Labour and Welfare of Japan. It is a voluntary cooperation scheme aimed at facilitating harmonisation on cosmetics regulations, as well as maintaining a dialogue with industrial associations from the US, EU, Canada and Japan. Directorate

[18] http://ec.europa.eu/consumers/sectors/cosmetics/cooperation-trade/international-level/index_en.htm

General Health and Consumers (DG SANCO) and the JRC are participating in this cooperation, the lead being with DG SANCO.

ICCR operates via regular teleconferencing amongst member organisations, cooperation results being summed up at a meeting taking place yearly in June-July. Discussion on topics of interest for cosmetics regulation is facilitated within the context of working groups, often formed jointly with representatives of industry and regulators, which report on their activities to ICCR at teleconferences and at the yearly meeting. The creation of ICATM was inspired in the ICCR context.

During the period 2010-2012, ICCR operated regularly under the rotating chairmanship of Canada (2010), the EU (2011), and the US (2012), with ICCR working groups covering in particular alternatives to animal testing, nanomaterials characterisation and safety, as well as traces of unwanted substances in cosmetics.

With specific emphasis on alternatives to animal testing, ICCR received via the JRC regular updates about the activities of ICATM, including a status report on Alternative Test Method Validation and Regulatory Acceptance issued on a bi-annual basis after the ICCR 5th meeting in Paris. While endorsing the June 2012 status report at their 6th meeting in Rockville (Maryland, USA)[19], the ICCR members acknowledged its usefulness for industry as a reference list of available validated *in vitro* assays and OECD Test Guidelines, and requested the European Commission to make it publicly available on the JRC Website[20]. At the same meeting, a white paper on the "Applicability of Animal Testing Alternatives in Regulatory Frameworks within ICCR Regions", jointly prepared by the industry associations of the four jurisdictions was approved and later posted on the website of the US FDA[21]. To further strengthen international cooperation on alternatives to animal testing, ICCR also agreed at the Washington meeting to establish a new ad hoc joint industry-regulators working group with the mandate of exploring the suitability of *in silico* approaches for assessing the safety of cosmetic ingredients (http://www.fda.gov/Cosmetics/InternationalActivities/ConferencesMeetingsWorkshops/InternationalCooperationonCosmeticsRegulationsICCR/ucm312833.htm.

The final output will be a high-level review of *in silico* capability that could then serve as a roadmap for the eventual application of *in silico* methods in the assessment of cosmetic ingredients.

5.4 The OECD Adverse Outcome Pathway development programme

Advances in toxicogenomics, bioinformatics, systems biology and computational toxicology are set to drive a paradigm shift in regulatory toxicity testing and risk assessment. At the heart of these rapidly emerging approaches is the generation and treatment of exceptionally large datasets that are rich with mechanistic information. Moreover, with the aim of understanding adverse health effects and the underlying mechanisms specific to human biology, and to avoid unnecessary and often uninformative testing on animals, modern experimental investigations are looking to the use of human derived cells treated and comprehensively analysed *in vitro*. However, in order to rationally assemble, manage and interpret complex datasets to distil out the relevant information useful for regulatory risk assessment, the OECD proposed a conceptual framework based on an analytical construct

[19] http://www.fda.gov/Cosmetics/InternationalActivities/ConferencesMeetingsWorkshops/InternationalCooperationonCosmeticsRegulationsICCR/ucm312833.htm

[20] http://ihcp.jrc.ec.europa.eu/our_activities/alt-animal-testing/alt_test_cosmetics

[21] http://www.fda.gov/downloads/Cosmetics/InternationalActivities/ConferencesMeetingsWorkshops/InternationalCooperationonCosmeticsRegulationsICCR/UCM320464.pdf

termed - Adverse Outcome Pathway (AOP). In essence an AOP provides a structured and formal means to explicitly describe and communicate knowledge on a specific toxicological process or mode of action, to which a group of chemicals can be associated. An AOP framework thus provides a means to explain toxicological effects on a theoretical level. AOPs serve a variety of purposes and can guide, for example, the design of integrated assessment and testing strategies, weight-of-evidence approaches for hazard assessment, and the judgement of the value and relevance of biological effect information derived from an *in vitro* or computational method.

After obtaining endorsement of the Joint Meeting and the WNT, in January 2013 the OECD officially launched the AOP Development Programme (AOPDP). Initially the Programme which will focus on the development and evaluation of AOPs that can be fed into other activity areas of the OECD such as the Test Guidelines Programme, the Task Force for Hazard Assessment, and the QSAR Toolbox Project. The management of the AOPDP has been entrusted to the OECD Extended Advisory Group (EAG) on Toxicogenomics and Molecular Screening. For a number of years the EAG has been active in bringing together key actors behind international research efforts in advancing toxicological science with the aim of translating the latest methods available for understanding and predicting toxicology to the domain of regulatory risk and safety assessment. This EAG is well qualified therefore to take on the responsibility for the AOPDP and is currently busy with defining a workplan that comprises projects covering AOP development, chemical-specific case studies, AOP related guidance documents (inc. reporting templates), and AOP related knowledge management tools (inc. IT platforms for community based collaboration).

Reflecting the Commission's commitment to assume leadership in pushing safety assessment science forward to lay a solid scientific foundation for the widespread development and use of alternative methods, the JRC/EURL ECVAM has taken up the co-chairing (with the US EPA) of the EAG and thus has a direct influence on the AOPDP itself. The JRC/EURL ECVAM is also contributing directly to the AOPD workplan through the undertaking of AOP development projects in the area of liver toxicity and co-leading a project in the establishment of a Mode of Action Knowledge Base, constructed using the AOP framework. Moreover, JRC/EURL ECVAM has also taken the lead of an OECD project to be conducted within the context of the Task Force on Hazard Assessment with the aim of developing an Integrated Testing Strategy (ITS) for skin sensitisation hazard classification (GHS) based on alternative methods. This is closely linked to the implementation of the EURL ECVAM strategy on the full replacement of animal testing for this endpoint, as communicated recently as a JRC Scientific and Policy Report.

5.5 References

National Research Council (2007). Toxicity Testing in the 21st Century: A Vision and a Strategy. The National Academies Press, Washington, DC,

6. The EURL ECVAM Validation Process

The EURL ECVAM validation process has been streamlined and standardised over recent years to adapt to new requirements and priorities. Today it can be summarised as follows:

The validation process starts with the receipt of test method submissions via the standard test submission procedure (*i.e.* through the EURL ECVAM website) or in reply to a specific call. The test methods are assessed by EURL ECVAM with regard to scientific and technical aspects, their regulatory relevance and impact on the 3Rs (*i.e.* replacement, reduction and refinement of animal use). For most complex health effects or 'endpoints' such as *e.g.* repeated dose systemic toxicity or carcinogenicity, the relevance of a single test method is typically evaluated with respect to its potential usefulness when combined with complementary methods, for example within an integrated testing strategy. Ideally, the test method is assessed within the framework of an EURL ECVAM strategy that has been defined for each toxicological area. These EURL ECVAM strategies typically address different regulatory domains and related needs, review the progress made to-date, identify gaps and opportunities in relation to method development and validation, and outline what actions should be taken to deliver solutions that carry 3Rs impact. While formulating these strategies EURL ECVAM usually consults with its regulatory and scientific advisory bodies, its stakeholder forum and with international cooperation partners also involved in method validation. In some cases EURL ECVAM also consults its advisory and stakeholder bodies regarding the merits of individual methods if deemed appropriate.

If the submitted test methods are found to be sufficiently developed and relevant for entering validation then a study is launched if suitable resources are available. A validation may be executed by third parties or in some cases the method is transferred to the EURL ECVAM laboratories where the test method protocols are reviewed and refined if necessary, in close collaboration with the test method submitters. If the validation study foresees a ring trial to demonstrate reliability of the method, then EURL ECVAM would first carry out a within-laboratory reproducibility study within its Good Laboratory Practice (GLP) compliant facility, followed by transfer (including training) to external laboratories. In future, such laboratories will be selected from the Network of Validation Laboratories (NETVAL) that will be formally established in 2013.
It is foreseen that the time needed for validating a test method will be reduced by thoroughly reviewing and better defining the submitted test method protocols early on and by transferring the protocols to the EURL ECVAM laboratory first before entering a multi-laboratory ring trial. In addition, the availability of a network of validation laboratories should also contribute to a reduction in time needed for the planning phase.

If the validation trial has been completed successfully, a validation study report is drafted and submitted to the ECVAM Scientific Advisory Committee (ESAC) for independent peer review. ESAC issues an opinion on the test method's scientific validity in context of its intended purpose. On the basis of the ESAC opinion and regulatory/stakeholder input, EURL ECVAM drafts its recommendation on the validated test method which then undergoes a public commenting round before being published on the EURL ECVAM website and communicated to Commission services and other stakeholders.

Further to the recommendation, EURL ECVAM may decide to lead on behalf of EC the regulatory acceptance process by drafting an OECD TG and an EU test method to be adopted in the EU test method regulation. In case another OECD member country takes that lead,

EURL ECVAM representatives are nevertheless usually actively participating in the different OECD expert groups who review new and updated TGs.

With the adoption of Directive 2010/63/EU on the protection of animals used for scientific purposes, ECVAM became the "European Union Reference Laboratory on alternatives to animal testing (EURL ECVAM)". Since 2012, it is part of the new Systems Toxicology Unit of the Institute for Health and Consumer Protection (IHCP) of the European Commission's Joint Research Centre. Its key responsibilities are to (1) guide and participate in the development of alternative methods, (2) conduct and coordinate validation studies, (3) facilitate regulatory acceptance and (4) promote the use of alternative methods by end-users.

7. Conclusions

Significant progress has been made in the validation and regulatory acceptance of alternative methods in the areas of skin irritation and corrosion, phototoxicity, eye irritation, and genotoxicity, which is resulting in a considerable reduction of animal use for these endpoints. For the more complex toxicological effects, the emphasis at the moment is on research and development based on understanding toxicology rather than simply observing it. The key is the use of mechanistic knowledge to rationally design integrated assessment and testing strategies that are fit for specific regulatory purposes. Emerging technologies and approaches, improved scientific understanding and a strong collective desire to move away from animal testing are driving a shift in the safety assessment paradigm. Validation strategies need to continually adapt to fit both with the nature of novel methods and the specific needs of the different regulated sectors. At global level, initiatives like the AOP programme at OECD, the Tox21 initiative in the US and the increased interest shown in alternatives within countries such as Brazil, China, Russia, India and South Korea keeps the hope and wish alive for a better and more predictive toxicology without the need to rely on animals.

Annex 1

Summary status of validation of alternative methods at EURL ECVAM

A summary status update on the validation of alternative methods at EURL ECVAM is provided in Table 1. This information covers the period 2010 until today (1st quarter of 2013). For previous updates one should refer to the ECVAM technical report 2008-2009 (Zuang *et al.*, 2010[22]).

Table 1. Status of validation of alternative test methods at EURL ECVAM since 2010

Nr.	Toxicity area	Test method description	Validation status
1	Carcinogenicity	Cell Transformation Assay (CTA) SHE	EURL ECVAM recommendation published in 2011
2		Cell Transformation Assay (CTA) Balb/C	EURL ECVAM recommendation published in 2011
3		Cell Transformation Assay (CTA) BHAS	ESAC peer review finalised
4	Skin sensitisation	KeratinoSens test method	ESAC peer review finalised
5		Direct Peptide Reactivity Assay (DPRA)	ESAC peer review finalised
6		human Cell Line Activation Test (h-CLAT)	ESAC peer review foreseen to start in 2013
7	Acute oral toxicity	3T3 Neutral Red Uptake (NRU) test method	EURL ECVAM draft recommendation to be published in April 2013
8	Toxicokinetics	Cytochrome P450 (CYP) induction assay using the human cryopreserved HepaRG® cell line and cryopreserved human hepatocytes	ESAC peer review foreseen to start in 2013
9	Eye irritation	Reconstructed human tissue model (EpiOcular™ EIT)	ESAC peer review foreseen to start in 2013
10		Reconstructed human tissue model (SkinEthic™ HCE)	ESAC peer review foreseen to start in 2013
11	Endocrine disruption	MELN® estrogen receptor transactivation assay (agonist and antagonist protocols)	ESAC peer review foreseen to start in 2013
12		Androgen receptor transactivation assay (agonist and antagonist protocols)	EURL ECVAM validation foreseen to start in 2013
13		Androgen receptor transactivation assay (agonist and antagonist protocols)	EURL ECVAM validation foreseen to start in 2013
14	Fish acute toxicity	Fish Embryo Acute Toxicity Test	ESAC peer review finalised

[22] Zuang, V. *et al*. (2010) ECVAM Technical Report on the Status of Alternative Methods for Cosmetics Testing. JRC Scientific and Technical Reports. ISBN 978-92-79-16021-9. Available at: http://ec.europa.eu/consumers/sectors/cosmetics/files/pdf/animal_testing/at_ecvam_2008-2009_en.pdf.

Summary status of the regulatory acceptance of alternative methods within the OECD Test Guideline Programme

A summary status update on the regulatory acceptance of alternative methods within the OECD Test Guideline Programme is provided in Table 2. This information covers the period 2010 until today (1st quarter of 2013). For previous updates one should refer to the ECVAM technical report 2008-2009 (Zuang *et al.*, 2010).

Table 2. Status of regulatory acceptance of alternative methods since 2010

Nr.	Toxicity area	Test method description	Acceptance status
1	Skin corrosion	Reconstructed human Epidermis test methods (RhE) as included in OECD TG 431/EU TM B.40 bis	Accepted in 2004, updated version (subcategorisation, performance standards, inclusion of SkinEthic™ RHE and epiCS®) adopted at WNT in 2013
2		Transcutaneous electrical resistance (TER) test as included in OECD TG 430/EU TM B.40	Accepted in 2004, updated version (performance standards) adopted at WNT in 2013
3	Skin irritation	Reconstructed human Epidermis test methods (RhE) as included in OECD TG 439/EU B.46	Accepted in 2010, updated version (performance standards, inclusion of LabCyte EPI-model) adopted at WNT in 2013
4	Eye irritation	Fluorescein Leakage (FL) test method as included in OECD TG 460	Accepted in 2012
5		Bovine Corneal Opacity and Permeability (BCOP) test method as included in OECD TG 437/EU TM B.47	Accepted in 2009, updated version (positive control, use in a bottom-up approach to identify non-classified chemicals) adopted at WNT in 2013
6		Isolated Chicken Eye (ICE) test method as included in OECD TG 438/EU TM B.48	Accepted in 2009, updated version (use in a bottom-up approach to identify non-classified chemicals) adopted at WNT in 2013
7		Cytosensor Microphysiometer (CM) test method	New draft TG discussed at WNT in 2013 but not yet adopted, pending further clarification on its use in a bottom-up approach
8	Carcinogenicity	Cell Transformation Assay (CTA) SHE	New draft TG discussed at WNT in 2013 but not yet adopted , need for a Guidance Document
9	Genotoxicity	Existing OECD TGs under revision	Draft OECD TG 473 (*in vitro* chromosome aberration assay) and OECD TG 487 (*in vitro* micronucleus test) will be discussed at WNT in 2014
10	Endocrine disruption	Estrogen receptor transactivation assay (BG1Luc ER TA; agonist and antagonist protocols) as included in OECD TG 457	Accepted in 2012
11		Performance-Based Test	Accepted in 2012

		Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists (OECD TG 455)	
12	Fish acute toxicity	Fish Embryo Acute Toxicity Test	New draft TG adopted at WNT in 2013

An overview of all test methods submitted to EURL ECVAM and the progress of those test methods which were prioritised for validation can be found in the Tracking System for Alternative methods towards Regulatory acceptance (TSAR; http://tsar.jrc.ec.europa.eu).

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Abstract
Provisions of Regulation No 1223/2009 on cosmetic products require that the European Commission reports on a yearly basis to the European Parliament and Council on the progress made in the development, validation and regulatory acceptance of alternative methods and on the compliance with the deadlines of the animal testing and marketing bans. This EURL ECVAM technical report provides an update since 2010 on the state of play of alternative methods for *all* the toxicological areas relevant to the Cosmetics Regulation and supplements the 2013 Commission Communication on the animal testing and marketing ban and on the state of play in relation to alternative methods in the field of cosmetics. Overall good progress has been made in the validation and regulatory acceptance in areas such as local toxicity where the underpinning science is more advanced and mature alternative methods are available. For very complex endpoints on the other hand, such as chronic systemic toxicity, carcinogenicity or reproductive toxicity , efforts are predominantly focused on research and development where the emphasis is on the integration of a variety of methods based on mechanistic understanding.. The future is bright however, since considerable advances in new *in vitro* technologies, systems biology, bioinformatics and computational toxicology are driving a paradigm shift in toxicological testing and assessment where non-animal methods will ultimately become the tools of choice.

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