

Exploring new approaches to assess safety without animal testing

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Abstract

The 7th Amendment to the EU Cosmetics Directive has made developing non-animal approaches to assure the safety of consumer products a key business need to support future innovation. A substantial research programme was initiated by Unilever in 2004, aimed at critically evaluating the feasibility of a proposed new conceptual approach. The key aspects of this approach are:

1. Developing new risk assessment approaches
2. Developing new computer-based (*in silico*) and biological (*in vitro*) models
3. Evaluating the applicability of new data-rich technologies for generating and interpreting information for risk-based safety assessment.

We have focussed initially on skin allergy (sensitisation). A new risk assessment framework has been defined and is under evaluation. In collaboration with Entelos Inc., we have developed an *in silico* model of the induction of skin sensitisation, to help guide our development of new *in vitro* assays and to provide the biological context for integrating different types of data. New *in vitro* models are being developed in-house and through various research partnerships, including via COLIPA and as part of the EU Framework 6 Programme multi-partner research project, *Sens-it-iv*. The applicability and integration of data from 'omics' technologies are being investigated, including the use of *Cytoscape* for constructing, visualising and interrogating biological networks in a collaboration with the University of California San Diego. Work is ongoing to evaluate the applicability of this new risk-based approach for skin allergy, and investment is being made in applying the approach to other consumer safety endpoints.

Keywords: *in silico*, *in vitro*, new technologies, risk assessment, skin allergy

Introduction

This paper outlines some of the new scientific approaches that are being evaluated by Unilever for their applicability for conducting safety risk assessments on the ingredients used in consumer products without having to generate new data in animal tests.

Decisions about the consumer safety of our products are made on the basis of a *risk assessment*, in which data on the potential hazards of the ingredients are interpreted in the context of the likely exposure to the product, i.e. the concentration of the ingredients in the product and how the product is used by consumers. Traditionally, much of the hazard data on chemicals have been generated by applying *technologies* developed for histological and clinical chemical analyses to animal *models*. Today improved non-animal *in vitro* and *in silico* models are becoming

increasingly available, and new technologies such as proteomics and bioinformatics approaches enable us to generate and interpret new types of non-animal data. However, developing ways to integrate and determine the relative importance of these data to enable risk-based safety decisions to be made represents a major challenge (Fentem *et al.*, 2004; US National Research Council, 2007).

EU Cosmetics Directive and the Status of Alternative Methods

Both the 6th and 7th Amendments to the EU Cosmetics Directive have stimulated considerable research into developing alternative approaches to assess consumer safety. An animal testing ban on chemicals to be used in cosmetics comes into effect in the EU in March 2009, linked to a marketing ban in the EU on products containing any ingredients that

have been tested in animals for acute toxicity, skin or eye irritation, and mutagenicity. For various repeat-dose toxicity tests, the EU marketing ban is due to come into effect in March 2013 (EU, 2003).

During the last ten years, we have been successful in achieving the regulatory adoption of several Three Rs methods. *In vitro* replacement alternatives for skin corrosion, phototoxicity and dermal absorption are now included in OECD testing guidelines, as are refinement and reduction alternative tests and testing strategies for skin sensitisation and acute toxicity, both for local (eye and skin corrosion and irritation) and systemic effects (Fentem, 2006). In April 2007, ECVAM's Scientific Advisory Committee (ESAC) endorsed the scientific validity of an *in vitro* replacement test for acute skin irritation, and this will now be proposed for regulatory adoption and implementation (ECVAM, 2007). There are some examples of the use of *in vitro* alternatives in the risk assessment process; for example, information derived from *in vitro* skin penetration tests can provide valuable input to risk assessments. However, many of the current replacement methods were designed and validated to produce data for hazard identification and the classification and labelling of chemicals, rather than to generate the information needed for risk assessment. This view was confirmed in a report from the EU's Scientific Committee on Cosmetic Products (SCCP) in June 2007 (SCCP, 2007), which concluded that: "the majority of alternative methods [are] only suitable for hazard identification of cosmetic ingredients, but not for their risk characterisation."

The availability of replacement alternatives as of August 2007, and their applicability for consumer safety risk assessments, are shown in Table 1. The major challenges clearly relate to the types of safety endpoints subject to the 2013 deadline, which provide data for risk assessments when there is likely to be significant systemic exposure to a chemical.

There has been a shift in thinking and approach to the development and application of alternative

methods during the past five years, with more emphasis on test batteries and testing strategies as a way to combine data from different *in vitro* and *in silico* tests to make decisions about chemical safety based on risk assessments (Combes *et al.*, 2003; EU, 2005). As we start to consider more complex issues, such as how we might assess the multitude of potential adverse effects following repeated systemic exposure to chemicals without animal testing, we are realising that the focus needs to change: from the direct replacement of a specific animal test to firstly defining the actual information required to make a safety decision, and then secondly determining how this could be generated without animal testing (Fentem *et al.*, 2004).

Embracing New Technologies

The new technologies that are being developed appear to offer significant opportunities for a step-change in the approaches used in the future to assess consumer safety (Fentem *et al.*, 2004). These new technologies include: 'omics' technologies, informatics, advanced analytical methods, and tissue engineering. Application of these tools and technologies for risk assessment should enhance the scientific basis of public health protection as well as enabling us to move away from animal testing (Fentem, 2006). This vision, and a strategy to deliver it, has been articulated in a recent report from the US National Research Council (NRC), commissioned by the US Environmental Protection Agency. In its Summary, the report states that: "Advances in toxicogenomics, bioinformatics, systems biology, epigenetics, and computational toxicology could transform toxicity testing from a system based on whole-animal testing to one founded primarily on *in vitro* methods that evaluate changes in biologic processes using cells, cell lines, or cellular components, preferably of human origin" (US National Research Council, 2007).

Unilever's R&D Activities: 2004-2007

Much of the research that Unilever scientists have been undertaking during the past four years supports the need to embrace, and further invest in, the strategy proposed by the expert committee (the Committee on Toxicity Testing and Assessment of Environmental Agents, CTTEA) that prepared the US NRC report (US National Research Council, 2007). We have been working to assess the feasibility, in practice, of a theoretical "conceptual approach" we published in 2004 (Fentem *et al.*, 2004). Taking skin allergy (sensitisation) as a case study, we are evaluating the applicability of various new technologies and non-animal models for consumer safety risk assessment. To underpin this, we have invested in developing new capabilities in, for example: (a) mathematical

Table 1. Availability of Replacement Alternative Methods for Hazard Identification and Risk Assessment

	Hazard Identification Test ¹	Consumer Safety Risk Assessment
2009²		
Acute toxicity	No	(other approaches)
Skin irritation	ECVAM validated method	Yes
Eye irritation	labelling severe irritants	Yes
Mutagenicity	Yes	testing strategy involves animal studies
2013²		
Skin Sensitisation	No	No
Repeat-dose toxicity	No	No ³
Toxicokinetics	n/a	No
Reproductive toxicity	No	No ³
Carcinogenicity	No	(very unlikely to perform)

¹*In vitro* replacement alternative methods have been adopted by regulators for skin corrosion, phototoxicity and dermal absorption.

²2009 and 2013 are the deadlines in the 7th Amendment to the EU Cosmetics Directive (EU, 2003).

³Data on systemic exposure are a key requirement, e.g. from dermal penetration studies.

ECVAM = European Centre for the Validation of Alternative Methods, n/a = not applicable

and computational modelling of biology; (b) tools for mapping and analysing biological networks; (c) 'omics' technologies; (d) biological and chemically-based models; and (e) data integration approaches.

The key "building blocks" of the conceptual approach that enable the safety (risk management) decision are: (a) risk assessment, (b) data interpretation and processing (translation), (c) models, and (d) technologies (Fentem *et al.*, 2004). It illustrates how we envisage being able to take safety decisions that adequately protect human health without animal testing, by comparing experimental biological data with relevant clinical data, since these technologies and models are being widely applied in clinical research.

It has taken over three years to generate experimental results worthy of sharing and publishing. Since September 2006, we have been discussing and presenting our research findings, as "work in progress" at various scientific meetings. In the area of skin allergy, we are starting to translate parts of the CTAAEA's vision (US National Research Council, 2007) into practical application.

Assessing the Feasibility of the Conceptual Approach – "Assuring Safety without Animal Testing (ASAT)"

The objectives of our research programme are to:

- develop new risk assessment approaches
- develop and apply new models for predicting adverse effects
- evaluate the usefulness of data for risk assessment from applying new technologies
- maximise the use of both new and existing data

The feasibility of the approach has been assessed in collaboration with research partners outside and inside Unilever, and we are working on different projects with about 20 US- and UK-based academic and contract research groups and informatics organisations.

Case study: Skin allergy

To ensure that our products do not induce skin (contact) allergy in consumers we currently use information on the concentrations of the ingredients in the product, and on how the product is used by consumers, together with data generated in the mouse local lymph node assay (OECD, 2002) to assess whether a chemical ingredient has the potential to cause skin sensitisation, and thus skin allergy, in humans. Our ultimate aim is to develop a scientifically robust new approach to enable us to perform risk assessments without the generation of new data in animal models.

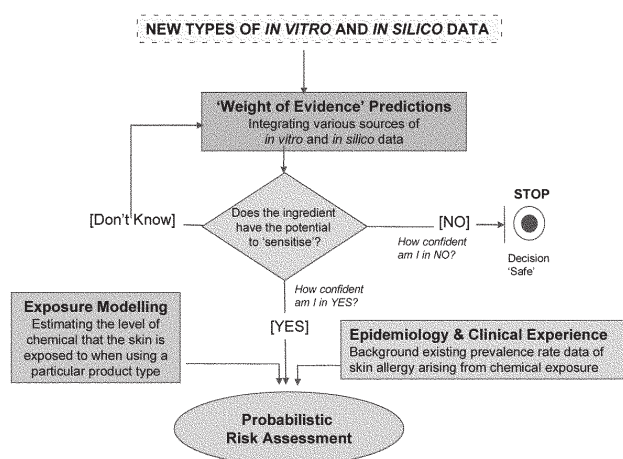


Fig. 1. New Risk Assessment Framework for Skin Allergy
A simplified version of the risk assessment framework defined for skin allergy. The major differences to the traditional approach are: the incorporation of new data inputs (*in vitro* and *in silico* information rather than data derived from animal studies), and the approaches being applied for modelling and integrating the data (*see text*).

Risk Assessment

The objective was to construct and evaluate a new risk assessment framework for skin allergy. The major changes are in the types of biological and chemical non-animal data inputs we are generating, and in the approaches being applied for modelling and integrating the data. We are also seeking to improve the data inputs on consumer exposure. A simplified version of the risk assessment framework we have defined is shown in Fig. 1.

In determining whether a chemical has the potential to induce sensitisation, *in vitro* data and *in silico* models are being generated to describe the key events considered to be important in skin sensitisation, based on our current mechanistic understanding. Recognising that our starting point is not a definitive set, based on our current mechanistic understanding the data inputs include: chemistry parameters, skin bioavailability, keratinocyte responses, protein-chemical reactivity, Dendritic cell activation and T-cell proliferation. Some relatively simple scoring approaches to integrating these data are being evaluated (Jowsey *et al.*, 2006), alongside the application of more complex approaches supported by various mathematical and informatics tools (*see Data Integration*).

To improve our current estimates of skin exposure to chemicals, we are applying probabilistic modelling approaches, such as Monte Carlo techniques, to understand the population distribution of exposure values rather than just taking mean estimates. This enables the refinement of our risk assessments, and makes the sources of uncertainty explicit. It is also possible that, for risk assessment approaches that do

not rely on data generated in animal models, more information on the local concentrations of chemicals and their flux through different skin compartments will be needed, in addition to the information on exposure used currently (i.e. dose applied to the skin surface).

Risk-based safety decisions also require detailed knowledge of the context and scientific background. For skin allergy, epidemiological and clinical data are valuable in providing this "real-life" context. Better understanding of the prevalence of skin allergy from exposure to specific chemicals, and the relationships between duration and frequency of exposure and the elicitation of clinical symptoms, will also improve our risk assessments. Similarly, it is important to improve our understanding of how the induction of skin sensitisation in the current mouse model relates to the elicitation of allergic responses in sensitised humans.

Models

Three examples of our current research into the development and application of new predictive *in silico* and *in vitro* models are outlined in this section. Firstly, to understand how systems biology approaches might be applied to support consumer safety risk assessments in the future, we have collaborated with Entelos, Inc. to construct a computer-based mathematical model of the induction of skin sensitisation (MacKay *et al.*, 2007). The vision was to build a transparent, robust, mechanistic and quantitative *in silico* model that captured our current understanding of the biological pathways, processes and mediators involved in skin sensitisation *in vivo*. The model has been constructed such that the biology can be interrogated computationally in an iterative, hypothesis-driven manner. The publications used in building and defining the biological relationships are both accessible and can be removed and replaced with other information. The benefits of the model are considerable, particularly as a way to integrate diverse data quantitatively and to interpret these within the wider biological context. We are now using the model to: (a) continue to generate new biological understanding; (b) guide our experimental research programme; (c) focus our development of new predictive *in vitro* assays; and (d) inform our risk assessments. Further development of the initial version of the model is in progress, via incorporation of new data from various biological and chemistry-based *in vitro* models.

Our work on *in vitro* modelling of the chemistry and biology of skin sensitisation complements research being undertaken by others, for example as part of the COLIPA research programme (COLIPA, 2007) and via the EU Framework 6 Programme integrated project, *Sens-it-iv* (*Sens-it-iv*, 2006). In particular, we

are developing tools to assess the covalent irreversible binding of chemicals to peptides underpinned by mechanistic classification of sensitisers based on their chemistry. We are trying to build upon the peptide binding assays currently available (Gerberick *et al.*, 2007), to incorporate kinetic, mechanistic and specificity considerations. Our development of new cell-based assays for allergenic potential has focussed on: (a) optimisation of Dendritic cell-based assays through addition of inflammatory signals and the identification of new biomarkers (e.g. intracellular signalling pathways); and (b) development of a robust *in vitro* T-cell proliferation assay, based upon a human Dendritic cell – T-cell co-culture model.

In the area of dermal kinetics, we are developing methods to study the detailed kinetics of permeation of chemicals through the heterogeneous layers of skin (Pendlington *et al.*, 2007). The standard *in vitro* skin penetration methodology described in OECD test guideline 428 (OECD, 2004) is being evolved to provide information on local concentrations of chemicals and their flux through different skin compartments. Time-course data on the epidermal / dermal disposition of chemicals (e.g. ¹⁴C-cinnamic aldehyde) in different vehicles in human split-thickness skin has been generated. These data enable the exposure information used in risk assessments for chemicals applied dermally to be refined, and provide valuable understanding of the effects of different vehicles and formulations on the dermal kinetics of chemicals.

Technologies

Our major research into the applicability of new technologies relates to gaining experience in generating, integrating and interpreting 'omics' data from various techniques, including transcriptomics, proteomics and metabolomics. The overall objective is to evaluate the applicability of 'omics' technologies, in combination, for generating data useful for consumer safety risk assessments. We selected skin inflammation as the clinical adverse response to investigate. Human volunteers are treated with sodium dodecyl sulphate (SDS) to induce a low-grade skin irritant response. Skin biopsies, interstitial fluid, blood and urine samples are then collected and analysed by using various analytical platforms: DNA microarrays (transcriptomics); liquid chromatography / tandem mass spectroscopy (proteomics); and gas chromatography / mass spectroscopy (metabolomics). Relevant functional data from cytokine and immunohistochemical analyses are also being generated. The intention is that the output of this work will provide a better understanding of the molecular mechanisms of skin inflammation, as well as practical experience in generating, integrating and interpreting very large and diverse data-sets of 'omics' and other *in vitro* data.

The management and analysis of the vast amounts of data generated in these experiments represents a major challenge and can be extremely time-consuming. We have developed an informatics platform to support the analysis and interpretation of these experimental data in an integrated manner. Working with the European Bioinformatics Institute, in-house databases have been built and federated to Web-based databases for adding further information about the biomolecules identified in our experiments. Working with the University of California San Diego, the open-source software *Cytoscape* (Shannon *et al.*, 2003) is being applied to integrate the data we are generating with biological network and pathway data. Preliminary results show that differences in erythema responses observed visually after skin patch testing with SDS correlate with, for example, the microarray gene expression profiles. It is anticipated that as further data are generated we will gain further insights into the molecular mechanisms of skin inflammation and be better able to understand how to use these new technologies in the future.

Data Integration

Our objective is to develop tools and approaches to integrate data of diverse types to facilitate their interpretation for consumer safety risk assessment. We are working to develop and evaluate three different kinds of approach: (a) "weight of evidence" methods, including simple scoring and more complex statistical (e.g. Bayesian) approaches; (b) *in silico* mechanistic modelling; and (c) biological network mapping and analysis of chemical-induced modulation of interactions between molecules in human systems.

Jowsey *et al.* (2006) have published an initial attempt to define a weight-of-evidence approach to integrate non-animal data inputs as surrogates for several key processes known to be important mechanistically in the induction of skin sensitisation. Whilst recognising that the list of assays or data requirements is not definitive, a conceptual approach for trying to integrate data via a simplistic scoring system has been outlined and is being evaluated. This is a pragmatic starting point given the limited availability of new types of data. As more data are generated, statistical (e.g. Bayesian) approaches will be used to model and interpret data in a more mathematically robust way.

The systems biology approach provides significant new opportunities for integrating and interpreting non-animal data in an overall biological context. The *in silico* model developed with Entelos gives us a biological framework and tools to begin to integrate many different types of data (MacKay *et al.*, 2007). It is the intention that our future research data, and those published in the scientific literature, will be incorporated into the model as we continue to develop

it as an integral part of our new non-animal approach to skin allergy risk assessments.

To make sense of much of the 'omics' data we are generating, and to be able to interpret them in a broader biological context, we have collaborated with scientists at the University of California San Diego in developing and applying the *Cytoscape* software. As a result, gene networks and human "interactomes" can now be constructed, visualised and analysed by applying a range of bioinformatics tools and systems biology approaches (Warner *et al.*, 2007).

Working together

The research being undertaken by Unilever is aligned with research we are also involved in with COLIPA, the European Partnership for Alternative Approaches to animal testing (EPAA) and the UK National Centre for the Replacement, Refinement and Reduction of animals in research (NC3Rs). We are active participants in the COLIPA research programmes on skin sensitisation, genotoxicity and eye irritation (COLIPA, 2007), and in trying to define a long-term research strategy for replacing animal tests for repeat dose systemic toxicity via the EPAA (EPAA, 2007). To discuss this specific challenge, several world class scientists from outside the field of toxicology (including Nobel prize-winning scientists) will participate in a workshop in April 2008 being organised by the EPAA.

Unilever scientists are also collaborating with the UK NC3Rs on its replacement and regulatory toxicology initiatives (NC3Rs, 2007). Tissue engineering for *in vitro* model development was a strategic research funding priority area for the NC3Rs in 2007. An NC3Rs' Regulatory Toxicology Forum has been established to promote the scientific research needed to develop non-animal models and risk assessment approaches, and to bridge the gap between scientific developments and regulatory acceptance. Amongst other projects, inhalation toxicity is being used as a case study to define exposure-driven strategies to reduce and replace animal testing.

Future perspective

The new Regulatory Toxicology Forum initiative from the UK NC3Rs (NC3Rs, 2007), along with the report from the US NRC (US National Research Council, 2007) referred to earlier, give us optimism for the future. Assuring safety without animal testing is a formidable challenge. The scientific evidence that has now been generated, by ourselves and other groups, indicates that our best hope for future progress and success in achieving this goal is to invest in applying the new technologies now available to us, in parallel with further defining and evolving the risk-based approaches we use to protect public health and the environment.

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