



MS8: Industry activities

Assuring Safety without Animal Testing: Unilever's Ongoing Research Programme to Deliver Novel Ways to Assure Consumer Safety

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Summary

Assuring consumer safety without the generation of new animal data is currently a considerable challenge. However, through the application of new technologies and the further development of risk-based approaches for safety assessment, we remain confident it is ultimately achievable. For many complex, multi-organ consumer safety endpoints, the development, evaluation and application of new, non-animal approaches is hampered by a lack of biological understanding of the underlying mechanistic processes involved. The enormity of this scientific challenge should not be underestimated.

To tackle this challenge a substantial research programme was initiated by Unilever in 2004 to critically evaluate the feasibility of a new conceptual approach based upon the following key components:

- 1. Developing new, exposure-driven risk assessment approaches*
- 2. Developing new biological (in vitro) and computer-based (in silico) predictive models*
- 3. Evaluating the applicability of new technologies for generating data (e.g. "omics", informatics) and for integrating new types of data (e.g. systems approaches) for risk-based safety assessment*

Our research efforts are focussed in the priority areas of skin allergy, cancer and general toxicity (including inhaled toxicity). In all of these areas, a long-term investment is essential to increase the scientific understanding of the underlying biology and molecular mechanisms that we believe will ultimately form a sound basis for novel risk assessment approaches.

Our research programme in these priority areas consists of in-house research as well as Unilever-sponsored academic research, involvement in EU-funded projects (e.g. Sens-it-iv, Carcinogenomics), participation in cross-industry collaborative research (e.g. Colipa, EPAA) and ongoing involvement with other scientific initiatives on non-animal approaches to risk assessment (e.g. UK NC3Rs, US "Human Toxicology Project" consortium).

Keywords: risk assessment, skin allergy, cancer, Colipa, NC3Rs, European Commission, EPAA

1 Introduction

Unilever's commitment to eliminating animal testing has been underpinned by our scientific research programme since the 1980's in developing and using alternatives to animal tests. Since 2004, we have invested an additional € 3 million a year towards the development of novel non-animal approaches to assure consumer safety. Unilever's conceptual framework for safety assurance is risk-based rather than hazard-based, meaning all available data on a new ingredient (including predicted levels of consumer exposure

during product use) are analysed using a weight-of-evidence approach to judge the level of risk prior to generating any new data to further characterise the hazard.

Our research efforts are currently focussed on the development and evaluation of new risk assessment approaches for assuring consumer safety in the areas of skin allergy (Maxwell et al., 2008) and cancer, and consideration of how risk-based approaches and the application of new models and technologies could be applied in the area of general toxicity (where we have used inhaled toxicity as a specific case study).



The scientific and technical challenges associated with assuring consumer safety without any animal testing in the areas outlined above are enormous and it is clear that no single research group or company will achieve these goals alone. For this reason, in order to develop alternative approaches for consumer safety, Unilever has its own in-house research programme and in addition works in partnership with a number of external groups. These partnerships include sponsoring research with academic institutions, investigating new approaches with contract research organisations, initiating bespoke research with biotechnology companies, and consultancies with key experts. In addition to our internal research programme we are also involved in EU-funded projects, e.g. Sens-it-iv, Carcinogenomics, we participate in cross-industry collaborative research, e.g. The European Cosmetics Association (Colipa) and the European Partnership on Alternative Approaches to Animal Testing (EPAA), and have ongoing involvement with other scientific initiatives on non-animal approaches to risk assessment, e.g. UK National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs), US “Human Toxicology Project” consortium looking to follow up the 2007 report from the US National Research Council (NRC, 2007).

2 Skin allergy

Following the principles of the conceptual framework (Fentem et al., 2004), we have been exploring the feasibility of delivering consumer safety risk assessment decisions for skin allergy that do not rely on data generated in animals (Maxwell et al., 2008). A key element of this work is to improve our current estimates of dermal exposure to ingredients for which we have detailed information on both formulation effects and consumer habits and use patterns. A new experimental approach based on *ex vivo* human skin has been investigated to determine skin compartmental concentrations and the delivery kinetics of a chemical *in vitro* (Pendlington et al., 2008). Techniques like this aim to establish a relationship between the exposure on the skin and the chemical bioavailability at the target site assumed to be the epidermis and dermis. However, the true bioavailability of free chemical in the skin tissue is also influenced by other parameters (e.g. skin metabolism, tissue adsorption and clearance mechanisms) and consequently ongoing research in these areas should ultimately provide more valuable information for novel risk assessments.

To determine whether a chemical has the potential to induce skin sensitisation many groups are currently working on non-animal predictive models to encompass the events which are considered to be key to the induction of skin sensitisation (including large programmes of work at Colipa (Aeby et al., 2008) and within Sens-it-iv). To test and explore the relative contributions of individual biological pathways thought to be key to the induction of skin sensitisation, we developed an *in silico* mathematical model (the “Skin Sensitisation PhysioLab[®]” [SSP] platform; Maxwell and MacKay, 2008) in collaboration with Entelos[®] Inc. The aim of this project was to determine the

key biological pathways that drive the sensitisation response by mechanistic modelling of the biology that has been reported as relevant in the scientific literature. The technique provided a systematic approach for the identification of key pathways as well as the identification of knowledge gaps. This information has been used to focus our *in vitro* assay development on the pathways of importance (e.g. inflammatory signals in the epidermis) and has motivated some of our fundamental research studies, in particular, investigation of functional proteomics of skin proteins modified by sensitising chemicals.

Jowsey et al. (2006) hypothesised that no single non-animal approach could be envisaged to generate sensitiser potency information and proposed that multiple forms of non-animal data would be required for this purpose. Based on this hypothesis and our evaluation of the published skin sensitisation literature (by using the SSP platform), our current hypothesis is that the integration of some or all of the following categories of non-animal information, in the context of human exposure, should yield a new measure of skin sensitiser potency: Chemical reactivity; epidermal disposition (or bioavailability); epidermal inflammation; dendritic cell activation; T-cell proliferation. For example, it is generally understood that any chemical (or metabolite derived from it) must form a stable (covalent) adduct with protein in the skin in order to stimulate an immune response (Divkovic et al., 2005). Consequently, the covalent modification of a protein by a reactive chemical (haptentation) is considered to be a key step in the induction of skin sensitisation. Several *in chemico* assays for measuring the extent and nature of chemical reactions with model peptides are being developed, underpinned by this hypothesis (e.g. Aeby et al., 2008; Gerberick et al., 2007; Natsch et al., 2007; Kato et al., 2003). For the purposes of deriving the maximum qualitative and quantitative information on the reactivity of a chemical with peptides for a non-animal skin allergy RA framework, we have developed an *in chemico* peptide reactivity profiling assay, which uses a panel of six single-nucleophile peptides (generic sequence AcFAAXAA, where X = Cys, Lys, Tyr, His or Arg, with H₂N-FAAAA representing the N-terminal nucleophile) with the aim of determining the reactivity profile of a chemical with a high level of confidence. We have now standardised this approach and tested 36 chemicals; varied patterns of reactivity reaction mechanisms which are not always theoretically foreseen have been observed (Aleksic et al., 2009).

Due to the increasing complexity of datasets from this and other *in vitro* approaches, statistical tools (including principle component analysis, partial least squares and generalised linear models) are being used to analyse data and establish the predictive capacity of each assay alone and in combination. A network approach is also being used to integrate the data in a probabilistic and biologically relevant manner by drawing on the pathway structure modelled in the SSP. The aim is to determine the feasibility of such approaches for providing hazard data for risk assessment. Currently animal data such as local lymph node assay data is used to evaluate non-animal approaches. However, such approaches will need to be evaluated within a risk assessment and consequently more emphasis will need to be placed on human clinical experience of skin sensitisation to ensure that



any extrapolation of data from non-animal predictive models is appropriate. By maintaining the emphasis of new approaches on the human relevance we can move beyond animal replacement and towards better risk assessment methods in skin allergy.

3 Cancer

Like skin allergy, the prevention of cancer from the use of consumer products represents an extremely important safety endpoint. Past strategies have relied heavily on results from *in vitro* tests (genetic toxicology tests) being confirmed by “definitive” animal studies (genetic toxicology and carcinogenicity tests). We believe that a new non-animal strategy can be developed that is more informative and ensures safety to the consumer.

Since March 2009, as a consequence of the 7th Amendment to the EU Cosmetics Directive (EC, 2003) there has been a ban on the genetic toxicology-based animal testing of chemical ingredients intended for use in cosmetic products in Europe. In practical terms this has meant the cessation of several genetic toxicology tests, including the widely used bone marrow micronucleus assay in rodents.

In vitro-only genetic toxicology assay strategies have a high irrelevant positive rate (i.e. positive results will be obtained for chemicals that are not carcinogenic (Kirkland et al., 2005)), and many common food-based biochemicals can be erroneously rejected (e.g. flavonoids) if *in vitro* regulatory tests are employed alone. This is because of the inherent nature of the current assays, and much on-going research is focussed on identifying approaches to increase the specificity of currently available *in vitro* genetic toxicology tests (e.g. a large research programme at Colipa). Because these tests are used purely in a hazard identification mode – the label of “genotoxicity” indicated by the current tests necessitates the rejection of that chemical if no follow-up testing is conducted. However, greater evidence and wider acceptance of the existence of thresholds for genotoxic events, determined in *in vitro* systems, is emerging (Carmichael et al., 2009). We believe this may provide a way forward for the risk assessment of new chemicals and we are utilising high-throughput methodologies, such as automated micronucleus scoring (Diaz et al., 2007), to provide the data necessary for low dose determinations of thresholds, in standard and newly engineered cell lines.

The characterisation of a material as “low-dose thresholded” will, however, require adequate understanding of the molecular mechanism of action of carcinogens. “Omics” technologies offer real hope in this regard. Successes with transcriptomics and metabolomics have shown discrimination between chemicals with probable thresholded characteristics, based on mechanistic understandings (e.g. the activation of DNA repair pathways, changes in the cell cycle and oxidative/metabolic stress). Major programmes of research, in the EU and US (e.g. Carcinogenomics and ILSI-HESI programme) will continue this trend.

Novel insights are being generated that will be capable of informing a risk-based approach and, through collaborative work, we are investigating several other new technologies to increase our understanding of the complex interactions that occur in

biological systems in response to carcinogenic ingredients. For example, technology from the field of biophysics (i.e. infra-red micro-spectroscopy is proving to be valuable in mapping and understanding the transformation of Syrian Hamster Embryo cells in culture, in response to chemical carcinogen exposure (Walsh et al., 2009)). Furthermore, work at MIT, Boston is providing new ways to interpret the complex pathways and interactions involved in eukaryotic responses to carcinogenic chemicals using specific gene-deleted libraries teamed with systems biology tools such as Cytoscape.

The challenge ahead will be to integrate these data to allow risk assessment to be performed for new chemicals in consumer products under the conditions of use. The application of systems biology approaches to anchor the *in vitro* measurements to relevant biomarkers and pathology pathways will be key in this regard and we are conducting research with partners at Barts and The London School of Medicine, UK in order to provide the much needed, greater molecular understanding of the processes that lead to human skin tumours.

4 General toxicity

The assurance of consumer safety for novel ingredients without the generation of new animal data still remains a considerable scientific challenge, but in light of the progress described above towards new risk assessment frameworks for skin allergy and cancer we remain convinced that this is ultimately achievable. A major challenge for the future is how risk assessments for systemic toxicity may ultimately be performed in the absence of animal testing. We are currently working on understanding the work necessary in each of the following areas:

- Developing new, exposure-driven risk assessment approaches
- Developing new biological (*in vitro*) and computer-based (*in silico*) predictive models
- Evaluating the applicability of new technologies for generating data (e.g. “omics”, informatics) and for integrating new types of data (e.g. systems approaches) for risk-based safety assessment.

Key to progressing the development of new risk assessment strategies is the identification of the adverse health effects (and underlying mechanistic understanding of these health effects) that we are aiming to prevent in our consumers (a fundamental reason for the current progress that is being made in the areas of skin allergy and cancer). We have used a case study of inhalation toxicology to begin to understand how non-animal based approaches may be integrated for risk assessment purposes. This research includes (i) the development of a new exposure-based waiving approach for certain chemical classes (Carthew et al., 2009), (ii) exploration of the molecular understanding of mechanistic divergence between adverse and non-adverse effects (e.g. Carthew et al., 2006) and (iii) development of cellular models that will allow adverse lung effects to be predicted (e.g. Grainger et al., 2009)

New technologies are rapidly emerging that could offer the potential for ground-breaking opportunities in developing novel



ways to assess consumer safety (Fentem and Westmoreland, 2007). Advances in new technologies, particularly during the past decade, have opened up new avenues to the possibility that non-animal approaches could be achievable at some point in the future for human adverse effects more complex than local skin and eye irritation. For example, we have begun to explore potentially relevant new technologies in the areas of human tissue engineering (e.g. artificial lymph nodes), relevant cell-based approaches, “omics” technologies (transcriptomics, proteomics and metabolomics (which we have investigated in the context of skin inflammation), bioinformatics, advanced analytical methods, computer modelling (including systems biology) and new data interpretation/integration algorithms. Combined application of these tools and technologies in complementary and integrated ways should provide an enhanced scientific and increasingly more mechanistic basis for consumer safety assessment as well as enabling us to move away from animal testing to more human relevant analyses. The management and analysis of the vast amounts of data generated from “omics” experiments represents a major logistical and technical informatics challenge. Analysis can be extremely time-consuming and requires specialist bioinformatics capabilities. We developed a new 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) has been applied to integrate the data generated with human biological network and pathway data.

A wider vision and a shared strategic view of incorporating data from new models and technologies into potential novel frameworks for human safety testing has been articulated in a 2007 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” (NRC, 2007). Recently, the US “Human Toxicology Project” Consortium has been established to facilitate the global implementation of this NRC vision on toxicity testing for the 21st century.

Likewise within Europe, the EPAA have considered what approaches may be needed to address repeat-dose toxicity without animals, and a recent report in 2008 concluded “The time is right to harness more effectively the very substantial achievements that have been witnessed in biology and chemistry during the last 10 years. Many seminal discoveries and technological advances have the potential to impact substantially on the development of alternative approaches. Funding at the nexus of the disciplines of toxicology, biology chemistry and mathematics was recommended” (Anon, 2008). In 2009, the European Commission launched a Call for Proposals including funding

from Colipa for the development of a strategy towards alternatives to safety tests using animals in the area of repeat dose systemic toxicity. This includes research in the areas of advanced organ-simulating devices, novel methods to achieve functional differentiation of human-based target cells *in vitro*, optimisation of computational modelling and estimation techniques and integrated data analysis (EC, 2009).

A key area of new technology beginning to be investigated in the context of human safety is biological tissue engineered models derived from human primary cells, cell lines and stem cells (Westmoreland and Holmes, 2009). Organotypic models are very much in their infancy of development and, before any study of more complex human adverse effects in relation to chemical insult can begin, we must understand for what steps of human biological adversity we are trying to build the models. It may be possible to deconstruct and model some aspects of key multi-component pathways of complex adverse effects, e.g. using similar principles most commonly found in other areas of engineering. The analogy of mechanical engineering, however, is built upon an existing understanding of the component parts of the machine and its mechanism. However, we have a conundrum in human safety in that the first complex problem is identifying what the key pathways or components are for any interpretable adverse effect in order to be able to model it in a systems approach. We know the organs of the human body, but we do not often know the mechanisms of adversity in response to chemical insult. We cannot use randomly selected *in vitro* models to begin generating data as they may not be at all relevant to human effect. This aligns well with the NRC vision, which calls for a shift to a toxicity pathway-based paradigm for chemical risk assessment that holds great promise to be quicker and more predictive of human outcomes, including dose response modelling utilising computational systems biology models of the circuitry underlying each toxicity pathway (Andersen and Krewski, 2009).

The results from our research programme to date confirm our belief that an essential aspect of future success will be to involve multidisciplinary teams from all aspects of relevant new technology early in defining the strategy for addressing the best practical ways forward for exploring novel ways to assure consumer safety for complex safety endpoints.

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