Comment

Non-animal Replacements for Acute Toxicity Testing

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Summary — Current approaches to predicting adverse effects in humans from acute toxic exposure to cosmetic ingredients still heavily necessitate the use of animals under EU legislation, particularly in the context of the REACH system, when cosmetic ingredients are also destined for use in other industries. These include the LD50 test, the Up-and-Down Procedure and the Fixed Dose Procedure, which are regarded as having notable scientific deficiencies and low transferability to humans. By expanding on previous *in vitro* tests, such as the animal cell-based 3T3 Neutral Red Uptake (NRU) assay, this project aims to develop a truly animal-free predictive test for the acute toxicity of cosmetic ingredients in humans, by using human-derived cells and a prediction model that does not rely on animal data. The project, funded by Innovate UK, will incorporate the NRU assay with human dermal fibroblasts in animal product-free culture, to generate an *in vitro* protocol that can be validated as an accepted replacement for the currently available *in vivo* tests. To date, the project has successfully completed an assessment of the robustness and reproducibility of the method, by using sodium lauryl sulphate (SLS) as a positive control, and displaying analogous results to those of the original studies with mouse 3T3 cells. Currently, the testing of five known ingredients from key groups (a surfactant, a preservative, a fragrance, a colour and an emulsifier) is under way. The testing consists of initial range-finding runs followed by three valid runs of a main experiment with the appropriate concentration ranges, to generate IC50 values. Expanded blind trials of 20 ingredients will follow. Early results indicate that this human cell-based test holds the potential to replace aspects of *in vivo* animal acute toxicity testing, particularly with reference to cosmetic ingredients.

Key words: *acute toxicity, alternative method, cosmetics, fibroblasts, human-based, in vitro, LD50, Neutral Red Uptake, OECD, prediction model, REACH.*

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Introduction

Acute toxicity describes the adverse effects that occur following oral or dermal administration of a single dose of a substance, or multiple doses given within 24 hours, or an inhalation exposure of four hours (1). Following entry into the body *via* any of these routes, a reasonable early endpoint for the assessment of acute toxicity is the ability of the substance to cause basic toxicity (cytotoxicity) to human cells. For cosmetic ingredients, it is logical to test this initial cytotoxicity toward skin cells, which would be the early exposure site following dermal absorption. Previous studies have shown that basic cytotoxic effects are independent of cell type (2). Therefore, some scope exists for predicting the potential of a substance to cause similar damage at other body sites, based on the initial data from skin cells. The ultimate prediction model for acute toxicity would require the incorporation of ADME (Absorption, Distribution, Metabolism and Excretion) considerations, which characterise the fate of the substance in the body. In addition, organ-specific effects in differentiated cells at certain key sites of potential exposure (e.g. liver, kidney, nervous system) need to be considered. Such an approach may eventually incorporate multiple *in vitro* tests combined with other methods, such as computer modelling. The project described here aims to address the initial phase of basic cytotoxic effects of cosmetic ingredients following dermal exposure, by using human skin cells (dermal fibroblasts) as a relevant model for the initial local response, and careful extrapolation to the basic cytotoxicity likely to occur at other eventual exposure sites in the human body.

To date, the only regulatory tests for acute toxicity involve the use of animals. Methods such as the LD50, Up-and-Down Procedure and Fixed Dose Procedure are widely recognised as having

significant scientific flaws and low relevance to human toxicology (3, 4). In addition, ethical concerns and public pressure have driven the demand for scientifically and ethically advanced replacements — a sentiment embodied in recent legislation. On 11 March 2013, the European Union passed the Cosmetics Regulation (*Regulation 1223/2009*), which prohibits the testing of cosmetic products and their ingredients on animals (5). However, a new challenge has now arisen in the form of the REACH (Regulation, Evaluation, Authorisation and Restriction of Chemicals) system. While REACH is broadly supportive of replacement methods, it still advocates animal tests for some endpoints, including acute toxicity. This applies to cosmetic ingredients when they are also destined for use in other commercial products that are not subject to the Cosmetics Regulation. This contradiction between the Cosmetics and REACH regulations currently poses a major threat to the portfolio of ingredients available to the cosmetics industry, as well as to the innovation of new materials. An increase in the channelling of resources into the development of advanced animal-free testing strategies is urgently required.

Moving Forward

Significant time and financial resources have previously been invested in attempts to develop *in vitro* tests for human acute toxicity. These included studies by the European Union Reference Laboratory for the Validation of Alternative Methods (EURL-ECVAM) to implement the NRU (Neutral Red Uptake) assay with rodent 3T3 cells (6). While some progress was made, advances were limited by the use of animal cell culture systems and attempts to validate against animal data that is variable and not an appropriate benchmark for human toxicity. The current project aims to build on the positive aspects of previous work (7), while exploiting recent advances in cell culture technology, by using human dermal fibroblasts in animal product-free culture conditions and employing an innovative prediction model that does not use animal data. This ensures the creation of a fully human test as a basis for predicting human toxicity.

The project has received feasibility funding from Innovate UK (project number 131726), as part of the recent call for *Advancing the Development and Application of Non-Animal Technologies*, and runs from September 2014 to January 2016. The project partners are:

— XCellR8 (www.x-cellr8.com; lead partner): a GLP-accredited contract testing laboratory committed to the complete replacement of animal testing for the cosmetics, personal care and household product industries. All the experimental work will take place in XCellR8's laboratory.

— FRAME (www.frame.org.uk; Fund for the Replacement of Animals in Medical Experiments): a registered charity contributing its considerable experience in the development and validation of *in vitro* assays and providing independent data analysis.

— Lush (www.lush.co.uk): a leading global cosmetics company with a longstanding commitment to the full replacement of animal testing. Lush will play a key role in the selection of the panel of ingredients for the feasibility testing.

— Inventya (www.inventya.com): a leading innovation consultancy, which will support the project by conducting an in-depth market assessment and interview key stakeholders about the current status and future requirements for acute toxicity testing, in addition to supporting the dissemination and exploitation of the data.

The project partners have recognised the shortcomings in the current REACH requirement for the use of animal models in predicting human adverse effects, and have been actively working toward fully validated replacement techniques.

Twenty commonly used cosmetic ingredients, covering a range of expected toxicity levels and from a variety of functional categories (e.g. colours, fragrances, preservatives, surfactants, and UV filters), have been carefully selected to make up the testing panel for the feasibility study in the current project. The panel includes ingredients that are present in lip products and are therefore prone to ingestion during routine use. Three key test parameter goals have also been defined:

— To ensure that the test is *scientifically relevant*. This is facilitated by the combined expertise and experience of the project partners, and wider discussion with stakeholders through the market research activities.

— To guarantee that the test is *sufficiently robust*. It is of great importance that the protocol developed has strong statistical validity through carefully designed experimental methodology.

— To confirm that the test is *reliable and reproducible*.

These parameters are critical, as the collective aim is for the test to eventually become a recognised standard method for laboratories worldwide.

In summary, the current project involves: a) developing a new human cell-based *in vitro* test for the prediction of the acute toxicity of cosmetic ingredients in humans, based on the adaptation of previous methods that used rodent 3T3 cells in the

NRU assay (8); b) establishing an *in vitro* protocol, documented in an SOP, which meets the acceptance criteria for performance with human dermal fibroblasts; c) conducting a blind trial with 20 known cosmetic ingredients, in order to pre-validate the approach; and d) generating a database for potential adverse human reactions to the selected 20 chemicals, by using an innovative prediction model without reliance on animal data. The first testing stage of the project, with ten sodium lauryl sulphate (SLS) positive control runs, has been completed. A reference range of IC50 values has been generated — the IC50 for the positive control is one of the acceptance criteria for the test runs, and a general confirmation of the test protocol's successful construct. The acute toxicity of a panel of 20 cosmetic ingredients of varying toxicities will next be evaluated by using the optimised method.

Method

Sodium lauryl sulphate (SLS) was selected as the positive control, based on past experience and available knowledge of the substance. SLS was also used as the test sample in the first testing stage of the project, in order to optimise the method for later use with the 20 cosmetic ingredients.

Human dermal fibroblasts were obtained in a cryopreserved state from Lifeline Cell Technologies (Frederick, MD, USA), and thawed into culture in FibroLife™ Xeno-Free medium (an animal product-free medium containing human serum; Lifeline Cell Technologies). The cells were cultured according to the supplier's instruction documents and XCellR8's corresponding SOP, and maintained at 37°C/5% CO_2. They were sub-cultured in the exponential growth phase by using animal product-free reagents.

For the test, the cells were seeded into 96-well plates, and visually scored for confluence to ensure a 20–50% coverage on the day of treatment. They were scored again at the end of the treatment period, to ensure maximum 90% confluence during the exposure period. The solubility of the test sample was determined, where necessary, by using the procedure described in the Organisation for Economic Co-operation and Development (OECD) Guidance Document 129 (9). The cells were treated with eight different concentrations of each test sample, with six wells per treatment. After 24 hours, the cells were carefully washed to remove the test sample and then incubated with Neutral Red solution. The cells were then carefully washed to remove excess Neutral Red and the solubilisation solution added (50% ethanol, 1% glacial acetic acid, 49% tissue culture grade water). Neutral Red uptake by the cells was determined by reading the absorbance at 540nm with a spectrophotometer. The raw data were transferred to a pre-prepared, validated template which calculates the IC values and checks for the acceptability of the results against the defined acceptance criteria. The data were analysed independently of the testing laboratory, to ensure impartiality. The IC20, IC50 and IC80 values were each determined from the results of the test (IC = inhibitory concentration leading to the given percentage loss of viability, e.g. IC50 = the concentration required to cause a 50% loss of viability relative to negative controls).

Each ingredient sample test run will include an SLS control plate with a defined series of concentrations, in order to determine the IC50. For a run to be valid, the IC50 for SLS must be within the mean ± 1 SD of the historical set of 10 runs with this substance.

Results

Test development

The project has passed the first testing stage, having successfully completed the 10 SLS positive control runs and generated a reference range of IC50 values. The SLS results showed that the range of IC50 values for the human dermal fibroblasts in animal product-free culture (37.7µg/ml to 75.3µg/ml) was similar to that generated in historical studies with the mouse 3T3 immortalised cell line (27.2µg/ml to 64.7µg/ml). Various aspects of the protocol were amended, compared with historical studies, including the Neutral Red concentration and ingredient dilution factors.

The study with five known ingredients representing the key functional groups is under way, which, to date, is mirroring the successful results obtained with SLS. Variable toxicity between these five ingredients is also apparent, as would be expected.

Market research

Inventya have been undertaking a preliminary market assessment of the current status of acute toxicity testing, and the demand for a new scientifically advanced and ethically sound alternative to the traditional LD50 and related tests. Based on their initial reports, it is evident that there is significant interest in this project within the cosmetics industry. Chris Longmore, an innovation consultant at Inventya and involved in the research, stated that: *"When examining the commercial feasibility of the proposed non-animal test, the gathered data indicates a healthy market for the method to exploit. With the global cosmetics ingredients industry set to grow to a value of $26B by 2018 (BCC Research), and cosmetic manufacturers seeking to introduce novel*

ingredients annually, there is likely to be a continued demand for such a test method. Interviews with directors and researchers in both cosmetic and chemical manufacturing gave the opinion that such a test may be invaluable for meeting the current and future legislative burden in both sectors. During interviews, professionals repeatedly stated that complying with the REACH Regulation, the Cosmetics Regulation, and consumer ethical demands, is a huge challenge. Subsequently, the prevailing opinion is this test can confer a significant commercial advantage to businesses."

Discussion

Selection of the NRU test for this project was linked to its relevance to the mechanism of cell membrane damage from key cosmetic ingredients (including surfactants), as well as absorption across membranes. Moreover, it provides true indications of general acute toxicity that are comparable with previous studies on mouse 3T3 fibroblast cell lines (10). The results so far show that the uptake of Neutral Red by the human cells differs from that of the 3T3 cells, as has been previously shown (11), which drove the optimisation of the method for human cells in this project.

The NRU test measures a cell's capacity to actively transport the dye across the cell membrane and to retain this vital dye. Membrane integrity is a key indicator of cell viability. When viability is compromised, the capacity of the cell to transport and retain the dye is affected. This project utilises human dermal fibroblasts, since the skin is the first and largest tissue type exposed to cosmetics. Although, by definition, cosmetics should not penetrate the skin barrier, there are increasing demands for active ingredients (e.g. anti-wrinkle agents) that can penetrate the skin, and therefore would reach the dermal fibroblasts and potentially be absorbed into the bloodstream for further distribution around the body. It has also been established that interaction (*via* cell signalling) between dermal fibroblasts, epidermal cells and sensory nerves can modify the barrier capacity of the skin. *In vitro* models to examine this are currently being developed (12).

The prediction model will not use animal data, as the project aims to create a fully human test for human acute toxicity. Based on the prediction model for acute toxicity generated for the 3T3 cells by using the FRAME database of results (13), a similar approach is being devised for this set of results, when completed. It is anticipated that the model will define IC20 and IC50 ranges, and the ratio of these values is expected to correlate with acute toxicity levels *in vivo*.

Historically, it has not been possible, nor approved, to perform predictive toxicity screening tests without the use of animals or animal-based data. Now, however, with new technologies and techniques, in conjunction with increased overall scientific and ethical incentives, it is possible to generate results that are more translatable for human targets by using methods that are truly animal-free. The protocol described here explicitly reflects these advancements.

Market research has shown that there is a confirmed industry requirement for a regulatory *in vitro* test for human acute toxicity. The development of a test that can replace the need for the LD50 and related methods is long overdue. There is also a great need for more models that use human data to effectively correlate results without the need for extrapolation from animals, which has a high transferability risk. For industrial safety screening purposes, this test could prove to be crucial for product development, while providing a much more detailed understanding of the relative effects of a multitude of cosmetic ingredients.

In order for the test to be validated and ultimately accepted as an OECD Test Guideline, it must pass through a series of stages. These include:

— a feasibility study (i.e. the results of this initial project);

— blind multi-centre trials to demonstrate that the method is both reliable and transferrable between laboratories;

— pre-validation and validation trials co-ordinated by EURL-ECVAM;

— recommendation of the adoption of the method as an OECD Test Guideline by ESAC (the ECVAM Scientific Advisory Committee), if validation is successful; and

— publication of an OECD Test Guideline, finalising the method as a regulatory test.

This process currently takes many years (sometimes a decade or more) to complete. There is widespread agreement that strategies for accelerating this process are urgently required, in order for the availability of regulatory tests to keep pace with advances in *in vitro* technologies and to prevent the unnecessary continued use of scientifically obsolete animal tests. Meanwhile, once initial development and the early validation steps are complete, some cosmetics companies and ingredient suppliers may opt to use the test as part of a non-regulatory pre-screening strategy.

This project aims to not only lead to an improved screening output for acute toxicity, but also to showcase the potential for non-animal methods, coupled with better experimental design, thus promoting the drive toward a time when animals are no longer used for human scientific modelling. Ultimately, the new test could have positive implications for the scientific advancement of acute toxicity testing beyond the cosmetics industry.

References

1. UN (2011). *Globally Harmonized System of Classification and Labelling of Chemicals (GHS): Fourth Revised Edition,* 561pp. Geneva, Switzerland: United Nations Economic Commission for Europe.
2. Clothier, R., Dierickx, P., Lakhanisky, T., Fabre, M., Betanzos, M., Curren, R., Sjöström, M., Raabe, H., Bourne, N., Hernandez, V., Mainez, J., Owen, M., Watts, S. & Anthonissen, R. (2008). A database of IC50 values and principal component analysis of results from six basal cytotoxicity assays, for use in the modelling of the *in vivo* and *in vitro* data of the EU ACuteTox project. *ATLA* **36**, 503–519.
3. Rosenkranz, H.S. & Cunningham, A.R. (2005). Lack of predictivity of the rat lethality (LD50) test for ecological and human health effects. *ATLA* **33**, 9–19.
4. Rispin, A., Farrar, D., Margosches, E., Gupta, K., Stitzel, K., Carr, G., Greene, M., Meyer, W. & McCall, D. (2002). Alternative methods for the median lethal dose (LD50) test: The Up-and-Down procedure for acute oral toxicity. *ILAR Journal* **43**, 233–243.
5. Anon. (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* **L324**, 22.12.2009, 59–209.
6. Prieto, P., Cole, T., Curren, R., Gibson, R.M., Liebsch, M., Raabe, H., Tuomainen, A.M., Whelan, M. & Kinsner-Ovaskainen, A. (2013). Assessment of the predictive capacity of the 3T3 Neutral Red Uptake cytotoxicity test method to identify substances not classified for acute oral toxicity (LD50 > 2000 mg/kg): Results of an ECVAM validation study. *Regulatory Toxicology & Pharmacology* **65**, 344–365.
7. Spielmann, H., Balls, M., Dupuis, J., Pape, W.J., Pechovitch, G., de Silva, O., Holzhütter, H.G., Clothier, R., Desolle, P., Gerberick, F., Liebsch, M., Lovell, W.W., Maurer, T., Pfannenbecker, U., Potthast, J.M., Csato, M., Sladowski, D., Steiling, W. & Brantom, P. (1998). The International EU/COLIPA *In Vitro* Phototoxicity Validation Study: Results of Phase II (blind trial). Part 1: The 3T3 NRU Phototoxicity Test. *Toxicology in Vitro* **12**, 305–327.
8. Ceridono, M., Tellner, P., Bauer, D., Barroso, J., Alépée, N., Corvi, R., De Smedt, A., Fellows, M.D., Gibbs, N.K., Heisler, E., Jacobs, A., Jirova, D., Jones, D., Kandárová, H., Kasper, P., Akunda, J.K., Krul, C., Learn, D., Liebsch, M., Lynch, A.M., Muster, W., Nakamura, K., Nash, J.F., Pfannenbecker, U., Phillips, G., Robles, C., Rogiers, V., Van De Water, F., Liminga, U.W., Vohr, H.W., Wattrelos, O., Woods, J., Zuang, V., Kreysa, J. & Wilcox, P. (2012). The 3T3 Neutral Red Uptake phototoxicity test: Practical experience and implications for phototoxicity testing — the report of an ECVAM–EFPIA workshop. *Regulatory Toxicology & Pharmacology* **63**, 480–488.
9. OECD (2010). *Series on Testing and Assessment No 129: Guidance Document on Using Cytotoxicity Tests to Estimate Starting Doses for Acute Oral Systemic Toxicity Tests*, 54pp. Paris, France: Organisation of Economic Co-operation and Development.
10. Borenfreund, E. & Puerner, J.A. (1985). Toxicity alterations determined *in vitro* by morphological and Neutral Red absorption. *Toxicology Letters* **24**, 119–124.
11. Clothier, R., Willshaw, A., Cox, H., Garle, M., Bowler, H. & Combes, R. (1999). The use of human keratinocytes in the EU/COLIPA international *in vitro* phototoxicity test validation study and the ECVAM/COLIPA study on UV filter chemicals. *ATLA* **27**, 247–259.
12. Moore, P., Ogilvie, J., Horridge, E., Mellor, I.R. & Clothier, R.H. (2005). The development of an innervated epithelial barrier model using a human corneal cell line and ND7/23 sensory neurons. *European Journal of Cell Biology* **84**, 581–592.
13. Clothier, R.H., Atkinson, K.A., Garle, M.J., Ward, R.K. & Willshaw, A. (1995). The development and evaluation of *in vitro* tests by the FRAME Alternatives Laboratory. *ATLA* **23**, 75–90.