The Status of Alternative (*in vitro*) Approaches to Toxicity Testing

Before registering and marketing new products such as medicines, cosmetics, industrial chemicals or agro-chemicals, manufacturers must generate safety data. These data are assessed by regulatory (usually Government) agencies, to help identify potential health risks for humans or adverse effects on the environment, before it is decided whether a product is acceptable for release. While testing requirements vary depending on product type, its intended use and geographical region, there is a degree of similarity in the guidelines for regulating the safety of many new products.

Moving away from animal use

Tests performed in living animals (*in vivo*) were traditionally regarded as the "gold standard" for predicting toxicity to consumers and the environment. However, there are increasing efforts by many manufacturers to move away from using animal models in safety testing. These initiatives align with ethical concerns and with the principles of the 3Rs of animal research – Replacement, Reduction and Refinement – particularly as some toxicity tests use significant numbers of mice or rats and can cause distress. As well as being time-consuming and costly, standard *in vivo* testing provides limited information on how substances exert their effects. There is also the question of whether data from certain animal models is truly predictive of human responses.

In 2013, the European Union (EU) banned the marketing of cosmetics containing ingredients that have been tested on animals. As the EU is a major market base, this has effectively led to a worldwide ban on animal safety testing for cosmetics. This has given enormous momentum to the development of *in vitro* alternatives, which use human or animal tissues, organs or cells. These approaches can be quicker and cheaper to conduct than *in vivo* tests and, in some cases, may provide mechanistic insights, which may make predictions of toxicity (or "adverse outcomes") more accurate. However, it is not known whether any new cosmetic ingredients have been marketed in the EU since the animal testing ban, highlighting the complicated nature of moving away from traditional animal-based safety tests.

Overcoming the challenges associated with switching to alternative approaches

It can be difficult to relate results from *in vitro* studies using one or a small number of cell types to the biology of a whole organism, but efforts are being made to make *in vitro* assays more physiologically relevant by more closely mimicking the *in vivo* environment. Examples of how this may be achieved include varying the geometry of cell cultures (*e.g.* 3-D liver spheroids), incorporating movement (*e.g.* breathing-like contraction of lung cell cultures) and fluid dynamics (*e.g.* replicating the circulatory system). *In vitro* data cannot be directly compared to outcomes in *in vivo* settings, but *in vitro* to *in vivo* extrapolation (IVIVE) techniques are being developed in attempt to address this challenge – for example, the UK's National Centre for the Replacement, Refinement and Reduction of Animals in Research (NC3Rs) has funded research into how *in vitro* doses relate to likely human exposures.

As new methodologies have emerged over the past decade, new, more advanced *in vitro* systems beyond single-cell systems (*i.e.* monocultures) have proliferated. New approaches must usually

undergo a lengthy and costly validation process to ensure they are robust, reproducible and human relevant, particularly if data are being generated to satisfy regulatory requirements. *In vitro computer based* toxicity assays are already widely used within companies to prioritise compounds for further development and inform *in vivo* testing strategies, which can significantly reduce the number of animals used for safety testing.

Nonetheless, there remains a significant time lag between the development of new *in vitro* approaches and their acceptance by regulators in place of animal experiments, or alongside limited *in vivo* data sets. Very few *in vitro* toxicology tests have been accepted as replacements by regulators – examples include the Corrositex® *in vitro* membrane barrier test method for skin corrosion and the EpiDerm© model for skin sensitisation, developed by MatTek.

Multicellular models currently under development, but not yet validated for safety testing, include those for the human lung: bronchiolar or alveolar models, the gastro-intestinal tract and the brain (*i.e.* mini-brains - cerebral organoids). IVIVE techniques have not yet suitable for these systems to be adopted by regulatory agencies. However, work is being done to develop standardised *in vitro* protocols for specific test methods – one such protocol involves using the *in vitro* human micronucleus test to assess whether a cosmetic or product constituent in contact with skin can damage chromosomes.

The need for integration of new methods and harnessing existing data

It is becoming increasingly clear that effective *in vitro* safety testing will require the integration of data from multiple approaches, so batteries of complementary tests are being developed. For example, stand-alone genotoxicity tests have traditionally been conducted for DNA damage and mutations, rather than being integrated as a component of systemic toxicity tests. The field is now moving towards integrating multiple biological effects for more holistic safety assessment, including detecting chemicals with the potential to cause cancer. These approaches use both flow-based systems and image analysis to assess DNA damage in conjunction with cell cycle abnormalities, activation of cell signalling pathways (*e.g.* p53), changes to cell energy levels or the development of oxidative stress. Examples include next generation risk assessments.

In addition to in vitro advances, an increasing number of computer based methods (using *in vivo* and *in vitro* data) are being developed. Computer based methods are used during medicines development to predict solubility, kinetics or drug metabolism *in vivo*. These methods are increasingly successful, although their utility can be strongly influenced by the quantity and quality of data that was used to develop them. Additionally, if the data that a model is based on are not transparently available, it will be more difficult for industry to put the model into practical use, of for regulators and legislators to accept it's use in risk assessment.

In Summary

The EU's decision to ban animal testing for cosmetics led to a unique opportunity to advance the development of *in vitro* and computational methods for safety testing. It is envisioned that sophisticated human-relevant models, based on human cells or 3D tissues and computer methods will ultimately lead to more accurate assessment of the human and environmental risk associated with new products. There are still barriers to overcome in satisfying regulators that these approaches will meet their requirements. Nevertheless, where *in vitro* and *in silico* approaches are proven to be robust, reproducible *and* sufficiently human predictive, it is only a matter of time before they replace animal tests in toxicology testing strategies, and are increasingly widely accepted within regulatory guidelines.

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