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The usefulness of integrated strategy approaches in replacing animal experimentation

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Abstract

It is currently well accepted that in general, more than one method is necessary to allow the full replacement of an animal experimentation. These so called partial replacement methods can be used within integrated strategy approaches that combine different methods and information sources. A number of integrated strategy approaches were implemented within recent years in different areas of safety and regulatory toxicology. Moreover, latest advances in biomedical research and bioengineering provide a major opportunity to make use of *in vitro* human-based and/or three-dimensional complex models that can contribute to achieve more physiologically-relevant models. Examples herein describe currently existing integrated strategy frameworks aiming at full or partial replacement purposes and/or at gaining mechanistic insights. Furthermore, a general concept is provided on how 3R methods might be integrated in a strategy approach in order to ensure that animal experimentation is conducted only as a last resort.

Key words

- Integrated strategy approaches
- IATA
- DAs
- AOPs

REPLACEMENT METHODS AND ADVANCES IN BIOMEDICAL RESEARCH

Replacement methods are usually defined as methods which permit a given purpose to be achieved without conducting experiments or other scientific procedures on animals according to the 3Rs Declaration of Bologna from 1999. It is currently well accepted that more than one method is necessary to allow the full replacement of an animal experimentation. These so-called partial replacement methods are considered to be alternative methods that can partially replace an animal experiment, but requires the use of additional alternative methods as part of a strategy approach for achieving full replacement of the animal test [1].

The definition of a replacement method has considerable evolved from the original definition of Replacement by Russel and Burch from 1959, who considered for example that an experiment performed on an anaesthetised animal, which is killed under the anaesthetic at the end of the experiment, was a Replacement method. More recently, the NC3Rs has defined replacement methods as "accelerating the development and use of models and tools, based on the latest science and technologies, to address important scientific questions without the use of animals" (www.nc3rs.org.uk/the-3rs). The Swiss 3RCC in counterpart defines replacement methods as full or partial replacement methodologies, that are based on e.g., cultured cells (including primary cells), tissues and organs as well as the use of testing strategies that take into account existing *in vivo* data, *in vitro* methods, *in silico*/computational methods, physicochemical properties and non-testing data (https://swiss3rcc.org/2018/05/02/what-are-the-3-r).

Latest advances in biomedical research and bioengineering provide a major opportunity to achieve more physiologically-relevant human models. Their use within integrated strategies, combined with the knowledge of the underlying biological process, can contribute to a more mechanistic and predictive human science whilst decreasing the animal studies that may be necessary. Sistare *et al*. [2] report that the increasingly sophisticated *in vitro* humanized test systems, emerging computational models and novel translations biomarkers are being used to improve the ability to better predict drug induced liver injury, and make the development of safer drug candidates less dependent on animal studies. Malloy *et al*. [3] highlights the usefulness of using predictive approaches based on e.g. *in silico* and *in vitro* approaches, computational models, to help assessing the safety of chemicals.

A study conducted on the application of 3Rs in toxicological research in the pharmaceutical industry, showed that there are existing synergies between all the 3Rs, and in particular that *in silico*, *in vitro* and *in vivo* methods all hold the potential to contribute to the reduction of animal use [4]. Furthermore, Wolfensohn

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[5] showed that the collaborative multicentre research projects within the framework of the European Innovative Medicines Initiative (IMI) research programme can reduce the dependence on animal use in areas where it has normally been viewed as necessary. In particular, the collaborative platforms enabled the 3Rs to be addressed and optimized on multiple different levels, such as the selection of models to be tested, the protocols to be followed and the interpretation of results generated, leading to an overall reduction in the use of laboratory animals.

Finally, Piersma *et al*. [6] suggest that a transition to a mechanistically-based human-focused safety assessment is needed, which steps away from the traditional animal studies, and defines human biology as a new standard. However, further research is necessary to overcome the still existing gaps in scientific knowledge and technological limitations [7].

TESTING STRATEGY APPROACHES

The first concepts of using integrated approaches for the regulatory safety assessment of chemicals were developed already back in the late 90s and 2000s [8]. Recently, a transformation of the current way of conducting testing calls for the use of pathway-based approaches that capture the current understanding and the physiological mechanisms underlying toxicity [8]. Testing strategy approaches represent a logical manner to combine pathway-based and mechanistic assays and information sources [9]. In recent years, the systematic combination of several information sources has been implemented in several areas of regulatory and basic toxicology. Different types of strategy approaches may be used for regulatory and toxicology purposes as described here below:

- ***Integrated Approaches for Testing and Assessment (IATA***) may allow for full and/or partial replacement of animal testing as recommended for e.g. the assessment of skin irritation & corrosion and ocular hazard. An IATA is defined as an approach based on multiple information sources that integrates and weights all relevant existing evidence and guides the targeted generation of new data, where required, to inform regulatory decision-making regarding potential hazard and/or risk (OECD GD255, www.oecd.org/env/ehs/testing/series-testing-assessment-publications-number.htm). An IATA necessarily includes a degree of expert judgement, for example, in the choice of information sources and their weighting. Nevertheless, some of the IATA components, such as Defined Approaches to Testing and Assessment (see below), can be standardised (i.e. rule-based);
- ***Defined Approaches for Testing and Assessment (DAs)*** permit to derive a prediction that may be used on its own if they are deemed to be fit-for-purpose or considered together with other sources of information in the context of IATA to contribute to a regulatory decision. A number of DAs have been proposed for example for the assessment of skin sensitization. A defined approach consists of a fixed data interpretation procedure (DIP) (e.g. statistical, mathematical models) applied to data (e.g. *in silico* predictions, *in chemico*, *in vitro* data) generated with a defined set of information sources to derive a prediction (OECD GD255). In contrast to the assessment process within IATA, that necessarily involves some degree of expert judgment, predictions generated with defined approaches are rule-based;
- ***Adverse Outcome Pathways (AOPs)*** are based on mechanistic information and may contributes to a regulatory decision. An AOP describes a sequence of events commencing with initial interaction(s) of a stressor with a biomolecule within an organism that causes a perturbation in its biology (i.e., molecular initiating event, MIE), which can progress through a dependent series of intermediate key events (KEs) and culminate in an adverse outcome (AO) considered relevant to risk assessment or regulatory decision-making (OECD GD233, AOPWiki, https://aopwiki.org).

Although a clear-cut framework such as those existing for regulatory purposes does not exist in basic and biomedical research, if the combination of different information sources to study a certain mechanism and/or effect is a part of good scientific practices. When making a survey about the current gaps and opportunities for implementing the 3Rs in Switzerland it was found out that most researchers are using more than one approach in parallel, combining the use of non-animal and animal procedures (3RCC, personal communication). A total of 88% of the 103 respondents using *in vitro* models use them in combination with other approaches and 67% of responders work occasionally or frequently with animals. The survey was answered by a total of 176 Swiss respondents coming from academia (69%), non-profit organisations (13%), industry (10%), hospital/clinics (5%) and government or regulatory agencies (4%).

INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT

As reported by Eskes and Hofmann [10], for assessing the skin irritation and corrosion of chemicals, current internationally agreed approaches recommend the use of an IATA adopted as OECD Guidance Document 203 (OECD GD203). The IATA allows to replace or minimize, to the extent possible, the use of *in vivo* animal testing whilst ensuring human safety. It comprises in a sequential way: i) the use of existing *in vivo* and *in vitro* information, physico-chemical properties and non-testing methods; ii) a weight-of-evidence evaluation of the existing data and iii) if needed, the conduct of prospective testing. Although no single *in vitro* test method can cover the full range of skin corrosion and irritation responses observed in the traditional *in vivo* Draize rabbit test, the currently validated and adopted *in vitro* methods for skin irritation and corrosion can replace the *in vivo* test when combined within this recommended IATA. In particular, in Europe this IATA represents a full replacement of the animal testing as the regulatory adopted *in vitro* test methods can be used to distinguish non-classified chemicals. Animal testing is then conducted only when there is a need to satisfy other specific regulatory requirements, or when

the test chemical cannot be tested with the *in vitro* test methods currently adopted due to limitations or non-applicability.

In the area of eye irritation and serious damage, a number of *in vitro* test methods have been regulatory accepted for the identification of substances and mixtures that i) require classification for serious eye damage and ii) do not require classification for eye hazard effects [7]. However, there is currently no *in vitro* test method accepted for the identification of eye irritation, situated in the middle range of ocular hazard. Furthermore, although these assays may be combined in sequential testing strategies and/or within IATA as recommended within the OECD GD263, such strategies do not currently allow for the full replacement of the rabbit test for acute serious eye damage and eye irritation. One possible reason is that the *in vitro* test methods currently accepted to identify serious eye damage may not cover all mechanisms of action resulting in serious eye damage, including, in particular, the persistence of effects as observed *in vivo* at day 21 in the rabbit eye test method. This situation might lead to circumstances where an *in vivo* test method may need to be used as a last resort, in particular since persistence of tissue effects has been shown to be an important driver for the *in vivo* classification of serious eye damage.

The use of IATA and testing strategies have also been suggested for evaluating the carcinogenic potential of chemicals [7]. For the regulatory assessment of genotoxic effects of chemicals the use of a tiered testing strategy based on two *in vitro* assays is usually recommended. Carcinogenicity is then assessed based on a combination of genotoxicity study package and a two-year carcinogenicity study conducted in rats and mice. An IATA has been proposed to predict genotoxic carcinogenicity with high performance when using predictions falling within the model's applicability domain. For the non-genotoxic carcinogens an OECD expert working group is currently reviewing and assessing relevant *in vitro* assays with the aim of tentatively organise them into levels of testing, following an Adverse Outcome Pathway format, and examine how an IATA could be developed to assist regulators.

AOPs AND DAs FOR THE ASSESSMENT OF SKIN SENSITISATION

There is general agreement regarding the key biological events underlying skin sensitisation. The existing knowledge of the chemical and biological mechanisms associated with skin sensitisation has been summarised in the form of an AOP (OECD GD168), going from the molecular initiating event through the intermediate events up to the adverse health effect, i.e. allergic contact dermatitis in humans or contact hypersensitivity in rodents. The molecular initiating event (MIE) is the covalent binding of electrophilic substances to nucleophilic centres in skin proteins. The second key event (KE2) in this AOP takes place in the keratinocytes and includes inflammatory responses as well as gene expression associated with specific cell signalling pathways. The third key event (KE3) is the activation of dendritic cells, typically assessed by expression of specific cell surface markers, chemokines and cytokines. The fourth key event (KE4) is T-cell proliferation, which is indirectly assessed in the murine Local Lymph Node Assay (OECD TG429).

Three non-animal OECD Test Guidelines have been adopted addressing either the MIE, KE2 or KE3, and have used as a basis for developing IATAs and DAs. These approaches are mechanism-based, since they combine results from multiple test methods and/or computational tools that address different KEs of the AOP to assess the skin sensitization potential and in some cases potency of tested chemicals [11].

In particular, a number DAs and an IATA have been proposed for the purpose of skin sensitisation hazard assessment, potency categorization and risk assessment (OECD GD255 and GD256). These DAs not only vary in relation to the set of information sources used but also differ in the data interpretation procedures applied for converting the input data into a final prediction [12]. However, the data interpretation procedures have a common denominator that they are rule-based and do not require expert knowledge to derive a prediction, in contrast to an IATA. With a view to support the evaluation of integrated approaches in regulatory decision-making for skin sensitisation, the OECD is currently conducting a consistent and independent evaluation of the proposed DAs for skin sensitization. Human data, when available, and the LLNA as the only animal-based test that underwent formal validation, are generally used as reference for the assessment of animal-free test methods for skin sensitisation [13].

AOPs AND MECHANISTIC ONTOLOGY-DRIVEN STRATEGIES

The concept of adverse outcome pathways has also been applied within the US EPA's endocrine disruptor screening program. AOP has been used together with toxicity pathway frameworks to organize and integrate diverse biological data for evaluating the endocrine activity of chemicals [14]. The use of these frameworks allowed to establish biologically plausible links between endocrine mechanisms and apical responses when those endpoints are not measured in the same assay. The pathway frameworks facilitate a weight of evidence determination of a chemical's potential endocrine activity, identify data gaps, aid study design, direct assay development and guide testing strategies. The authors conclude that a variety of biological systems affect apical endpoints used in regulatory risk assessments, and without mechanistic data, an endocrine mode of action cannot be determined [14]. Furthermore, AOPs are proposed to reduce the reliance on long-term and costly fish early life-stage tests required for assessing the hazard of chemicals [15].

More recently, a mode-of-action ontology model for the evaluation of repeated dose toxicity of chemicals, has been proposed [16]. The authors report that a critical aspect in using non-animal approaches to assess the safety of chemicals is the challenge linked to the capacity to cover a comprehensive set of interdependent mechanisms of action, link them to adverse effects and

interpret their probability to be triggered in the light of the exposure to the chemical in question. Based on this, the authors propose critical elements and ways of establishing a mode-of-action ontology model to support the animal-free safety evaluation of chemicals.

Similarly, the use of an ontology-driven animal-free testing strategy has been proposed for developmental neurotoxicity testing, as a conceptual approach for designing testing strategies that cover the integral mechanistic landscape of developmental neurotoxicity [17]. Validation of these models require the coverage of the biological domain, rather than the classical predictive value of individual tests. The challenge is in mining modern biology, toxicology and chemical information to feed intelligent designs, which will define testing strategies for neurodevelopmental toxicity testing.

STRATEGY APPROACHES FOR NANOMATERIALS AND MEDICAL DEVICES

Due to the novel physicochemical properties of nanomaterials that are related to surface characteristics, the approach toward toxicity test development has distinct considerations from traditional chemicals and requires adaption of the existing approaches. Examples of strategies proposed for nanomaterials exist addressing the advantages and disadvantages of *in vitro*, *ex vivo* and *in silico* methods [18]. The authors also identify knowledge gaps for improving experimental and strategy design, highlighting the need to represent realistic exposure scenarios and to consider nanomaterials-specific concerns such as characterization, assay interferences and standardization.

In contrast, the US FDA/CDRH, discusses scientific principles, methods and endpoints for the replacement of conventional rodent testing by the inclusion and integration of clinical, diagnostic and pathologic data obtained from well-designed large animal studies. The recommendations include consideration for study designs that utilize methods for an overall more comprehensive interrogation of animal systems [19].

CONCLUSION

The progresses made in modern biology and bioengineering, call for a reconsideration on how to investigate and study the biological mechanisms that may lead to hazard effects, diseases, or to investigate the safety and efficacy of drugs. *Figure 1* depicts a diagram,

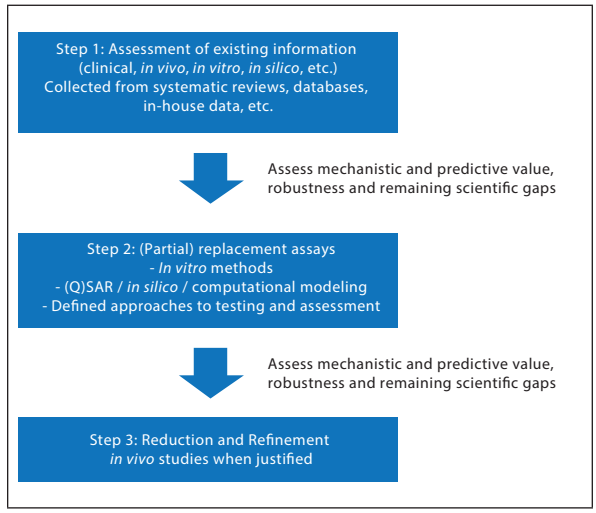

Figure 1
Conceptual framework for a strategy approach using Replacement, Reduction and Refinement alternative methods to animal experimentation, based on currently existing strategies used for regulatory toxicity assessment.

which takes into account the currently existing strategy approaches developed for regulatory purposes, and summarises the main steps that are usually undertaken based on the 3Rs Principle. The suggested steps within the diagram are not meant to be a prescriptive order. It is hoped that the above described examples of integrated strategy approaches used within the area of regulatory toxicology together with the overview given in *Figure 1* can contribute to other areas as to continue to promote the replacement, reduction and refinement of animal experimentation and ensure that animal procedures are used only as a last resort.

Disclaimer

The author contributed to this manuscript in her personal capacity. The views, thoughts and opinions expressed in this manuscript belong solely to her, and do not necessarily represent the views of the Swiss 3R Competence Centre

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